PhD-FSTC-2014-11
The Faculty of Sciences, Technology and Communication

DISSERTATION

Defense held on 13/05/2014 in Luxembourg
to obtain the degree of

DOCTEUR DE L'UNIVERSITÉ DU LUXEMBOURG

EN BIOLOGIE

by

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THE INTERACTION OF HLA CLASS I MOLECULES WITH THEIR RECEPTORS AND ITS EFFECT ON NK CELLS

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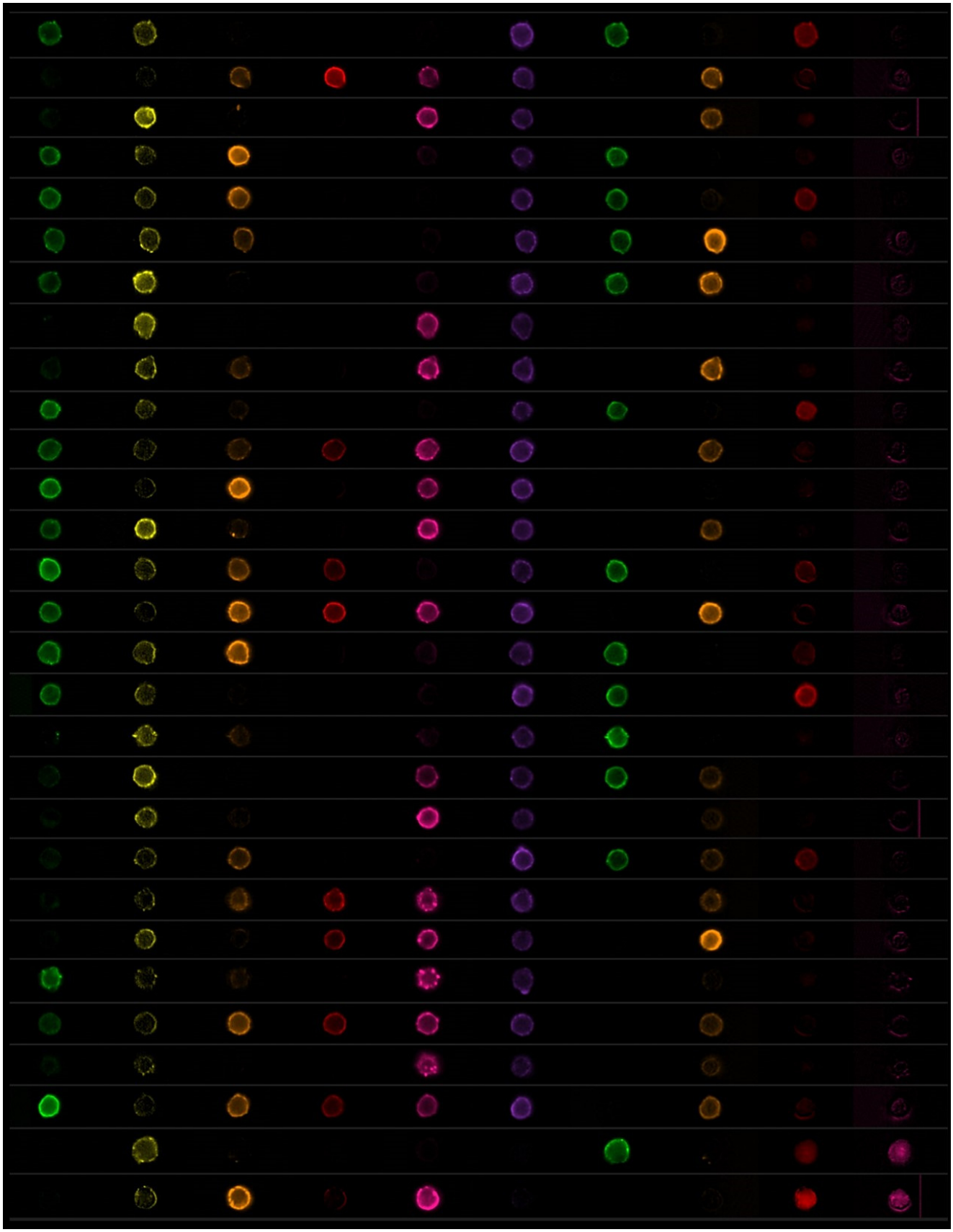

10-color staining of human healthy donor PBMC, gated for NK cells and measured on the ImageStream. Each line represents the expression of 10 different markers on a single cell. Image courtesy of Nicolaas H.C. Brons

To the greatest parents one could ever have,

To J. C. who inspires me every single day…

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ACKNOWLEDGEMENTS

This thesis is based on work performed at the Laboratory of Immunogenetics and Allergology (LIGA), Flow cytometry core facility (CFC) and the genomic research unit (GRU) at CRP-Santé Luxembourg, as well as collaborators in the Centre of Infectious Medicine (CIM), Karolinska Institutet, Stockholm, Sweden. I would like to express my gratitude to all those who contributed to the success of this project.

Dr. Jacques Zimmer, my thesis supervisor. I am really grateful for your support, encouragement and for believing in my abilities, and sharing your precious knowledge on NK cell biology. For all your patience and corrections during my thesis writing, thanks a lot.

Furthermore I would like to thank Dr Francois Hentges, head of the Laboratory of Immunogenetics and Allergology at CRP-Santé. For offering me the chance to proceed with my PhD thesis in his laboratory.

I am grateful to Nicolaas Brons (widely known as René! complicated story…) for sharing with me his endless expertise in flow cytometry, his knowledge in immunology and oncology, and for being a true teacher in and out of the laboratory. Also Maud Theresine for her great support and contagious optimism and smile, Stephanie Kler for her help in molecular biology, Aurelie Poli for her help in NK cell experiments, Francois Bernardin for his patience and advice (indeed he needed it when I had microarrays to perform) and Tony Kaoma for his availability and our endless discussions about bio-statistics.

I would like to thank all my colleagues in LIGA, Tatiana, Kyra, Tanja, Caroline, Olivia, Marion, Delphine, Christiane, Annette, Cathy, Angela, and Thorsten. As well as a big thank for Stephanie and Chantal for taking care of the mice.

Vivien Béziat, thanks a lot for all the experiments you performed as well as our discussions and exchanges.

I am more than grateful for all my colleagues who became real good friends and supported me in all possible ways; Maud Theresine, Victoria El-Khoury, Nassera Aouali, Angeliki Broukou, Gina Youssef, Sophie Rodius, Celine Jeanty, Alicia Dobosz, René Brons and Francois Bernardin, thanks a lot for making these four years pass so quickly.

I would never forget all my friends who stood next to me, for all our happy moments. Pascale Mustapha, Prune Viginier, Anne Maas, Severine DePoulain, Jerome Choffel, Franck Greff, Regis Singer, Sebastien Przeor and Marc-Olivier Thomas, the moments we spent together are unforgettable.

My mother Nada and father Charbel, no word can ever express how thankful I am for all your sacrifices and support in all my life choices. My sister Rawane and brother Jade, thank you guys for your encouragements although being far, and standing by me when I needed it most.

For all of you… THANK YOU

LIST OF PUBLICATIONS

Paper I (published)

NK cell KIR Repertoire Acquisition and Maturation are Strongly Modulated by HLA Class I Molecules

Sleiman M, Brons N.H.C, Kaoma T, Dogu F, Villa-Forte A, Lenoble P, Hentges F, Kotsch K, Gadola S.D, Vilches C. and Zimmer J.

The Journal of Immunology. 2014 Mar 15;192(6):2602-10

Brief Report I (in preparation)

HLA Class I Expression and Its Effect on NK Cell Gene Profile During Maturation and Cytotoxicity Mechanisms

Sleiman M, Kaoma T*, Bernardin F*, Brons N.H.C, Lenoble P, Vallar L. and Zimmer J.

* Contributed equally

In preparation

Letter I (published)

Clinical and Immunological Remarks about TAP Deficiency.

Zimmer J, **Sleiman M**, Hentges F. and Gadola SD.

Journal of Biological Chemistry. 2012 Aug 3;287(32):27047

Comment on:

Role of Metalloproteases in Vaccinia Virus Epitope Processing for Transporter Associated with Antigen Processing (TAP)-Independent Human Leukocyte Antigen (HLA)-B7 Class I Antigen Presentation.

Lorente E, García R, Mir C, Barriga A, Lemonnier FA, Ramos M. and López D.

Journal of Biological Chemistry. 2012 Mar 23;287(13):9990-10000

Letter II (published)

TAP Deficiency is Also a Cause of Bronchiectasis.

Zimmer J, **Sleiman M**, Poli A, Michel T. and Hentges F.

Thorax. 2013 May;68(5):490-1

Comment on:

Immunity and Bacterial Colonisation in Bronchiectasis.

Whitters D. and Stockley R.

Thorax. 2012 Nov;67(11):1006-13

ABREVIATIONS

aa	amino acids
ABC	ATP-Binding Cassette
ADCC	Antibody Dependent Cellular Cytotoxicity
ADP	Adenosine Diphosphate
AM	Adhesion Molecules
APC	Allophycocyanin
APCs	Antigen Presenting Cell
AR	Activating Receptors
ATP	Adenosine Triphosphate
BCR	B Cell Receptor
BLS	Bare Lymphocyte Syndrome
BM	Bone Marrow
BV	Brilliant Violet
CB	Cord Blood
CD	Cluster of Differentiation
CDC	Complement Derived Cytotoxicity
CG	Cytotoxic Granules
CLIR	C-type Lectin Inhibitory Receptors
CLP	Common Lymphoid Progenitor
CNX	Calnexin
CRT	Calreticulin
cSMAC	central Supramolecular Activation Cluster
CTL	Cytotoxic T Lymphocytes
Cy	Cyanine
DCs	Dendritic Cells
DMEM	Dulbecco's Modified Eagle Medium
DRiPs	Defective Ribosomal Products
EBV	Epstein-Barr Virus
eGFP	enhanced Green Fluorescent Protein
ER	Endoplasmic Reticulum
ERAAP	ER Aminopeptidase associated with Antigen Processing
ERAP1	ER aminopeptidase-1
FasL	Fas ligand
FcRγ	Fc Receptor Common Gamma Chain

FCS	Foetal Calf Serum
FITC	Fluorescein Isothiocyanate
HA	Hemagglutinin
HCMV	Human Cytomegalovirus
HD	Healthy donor
HHV-8	Human Herpesvirus-8
HIV	Human Immunodeficiency Virus
HLA	Human Leukocyte Antigen
HPV	Human Papilloma Virus
HPV	Human Papilloma Virus
HSCs	Hematopoietic Stem Cells
HSV	Herpes Simplex Virus
ICP47	Infected Cell Peptide 47
IDE	Insulin-degrading Enzyme
IFN	Interferon
Ig	Immunoglobulin
ILC	Innate Lymphoid Cells
ILT	Immunoglobulin-Like Transcripts
IR	Inhibitory Receptors
IRS	Inhibitory-Receptor Superfamily
ISIR	Immunoglobulin-Superfamily Inhibitory Receptors
ITAM	Immunoreceptor Tyrosine-based Activating Motif
ITIM	Immunoreceptor Tyrosine-based Inhibitory Motif
KIR	Killer-cell Immunoglobulin-like Receptors
KO	Knockout
KSHV	Kaposi's Sarcoma-associated Herpesvirus
LAMP	Lysosome-Associated Membrane Protein
LIR	Leukocyte Immunoglobulin-like Receptors
LN	Lymph Node
mAb	Monoclonal Antibody
MHC	Major Histocompatibility Complex
MM	Multiple Myeloma
mNK	mature Natural Killer
MTOC	Microtubule-organising centre
NBD	Nucleotide-Binding Domains
NK	Natural Killer

NKDI	NK Cell Developmental Intermediates
NWNA	Nylon Wool Non-Adherent
PB	Peripheral Blood
PBL	Peripheral Blood Lymphocytes
PBMC	Peripheral Blood Mononuclear Cells
PE	Phycoerythrin
PerCP	Peridinin Chlorophyll
PKC	Protein Kinase C
PLC	Peptide Loading Complex
PMA	Phorbol 12-myristate 13-acetate
pSMAC	peripheral Supramolecular Activation Cluster
PTK	Protein Tyrosine Kinase
PTP	Phosphotyrosine Phosphatases
rADCC	reverse Antibody Dependent Cellular Cytotoxicity
rhIL-2	recombinant human Interleukin-2
RPMI	Roswell Park Memorial Institute medium
SAP	SLAM-Associated Proteins
SLAM	Signalling Lymphocyte Activation Molecule
SLT	Secondary Lymphoid Tissue
SMAC	Supramolecular Activation Cluster
SNAREs	Soluble N-ethyleimide-sensitive factor Attachment protein Receptors
SNP	Single Nucleotide Polymorphysm
SYK	Spleen Tyrosine Kinase
TAP	Transporter Associated with Antigen Processing
TF	Transcription Factor
TGF	Transforming Growth Factor
TLR	Toll Like Receptor
TMD	Transmembrane Domains
TNF	Tumour Necrosis Factor
TPP II	Tripeptidyl Peptidase II
TRAIL	TNF Related Apoptosis-Inducing Ligand
Tsn	Tapasin
WHO	World Health Organisation
WT	Wild Type
β2m	β-2-microglobulin

ABSTRACT

The role of HLA class I molecule interaction with their receptors in NK cell education is well established, but the molecular mechanism behind it as well as the role of these ligands on their receptors acquisition during NK cell development, are still unclear. Trying to give more insight to these questions, two different studies were performed on NK cells derived from patients presenting a genetic defect in the transporter associated with antigen processing (TAP), leading to a strongly reduced surface expression of HLA class I molecules.

In our first paper, the expression of HLA class I receptors (three killer immunoglobulin-like receptors (KIR) - KIR2DL1/DS1, KIR2DL2/3/DS2 and KIR3DL1 - NKG2A, CD8) and the maturation marker CD57 were dissected in seven TAP-deficient patients and a panel of healthy donors (HD). A comparison of the two NK cell statuses (healthy and TAP-deficient) showed a significant increase in HLA class I receptors' co-expression in TAP-deficient compared to healthy NK cells. Functionally, hyporesponsiveness was confirmed for TAP-deficient NK cells: no cytotoxicity, degranulation nor IFN-γ production following co-culture with K562 cells. Only one patient presented functional NK cells with HLA class I receptors' co-expression pattern being different form HD's one.

In parallel, whole genome microarrays were performed on TAP-deficient *vs* HD's NK cells, aiming to identify the maturation (from $CD56^{bright}$ to $CD56^{dim}$) as well as cytotoxicity pathways which may be affected by HLA class I expression. The comparison of both statuses showed similar maturation processes (with different $CD56^{bright}$ and $CD56^{dim}$ gene profiles). Following 5 hours of $CD56^{dim}$ NK cells co-incubation with K562 cells, different gene clusters' evolution lead to identical final profiles contradicting the significant functional difference.

Taken together, our data demonstrate a strong effect of HLA class I molecules on NK cell maturation and functionality as well as on their KIR repertoire formation.

INTRODUCTION

1. Antigen Processing and Presentation

1.1. Introduction

During their lives, mammals are threatened by numberless pathogens (parasites, fungi, bacteria and viruses) as well as malignantly transformed cells. As a protection, evolution developed a sophisticated system of adaptive and innate immune cells, capable of differentiating the “self” from the “non-self” and defending the organism. This recognition is based on a turnover of cellular peptides which are presented on the surface of each nucleated cell by Major Histocompatibility Complex (MHC) class I molecules. The expression of MHC class I molecules is peptide dependent. Peptides resulting from the degradation of proteins by the proteasome are translocated into the Endoplasmic Reticulum (ER) lumen by the Transporter Associated with Antigen Processing (TAP). Once in the ER, these peptides are loaded on the MHC class I molecules by the Peptide Loading Complex (PLC), then processed to be expressed on the cell surface (Fig. 1).

In this first chapter, we will present the different steps implicated in the peptide processing and presentation, on the cell surface, by MHC class I molecules, with an emphasis on TAP protein, around which the PLC is centred.

1.2. MHC class I molecules

MHC class I molecules are expressed on the surface of almost all nucleated cells and play a very important role in the immune system function. Gene products encoded in the MHC complex region were first identified as being important in rejection of transplanted tissues. Different crystal structures of MHC class I molecules have been determined [14-17] showing the existence of two conserved domains, homologous to Immunoglobulin (Ig) constant region domains, which constitute the membrane-proximal region. The first one is a transmembrane glycoprotein with short cytoplasmic domains: the heavy chain, and the second is a soluble product of a non-MHC-linked gene: β_2-microglobulin (β_2m) (Fig. 2A). MHC class I genes exhibit enormous allelic polymorphism especially in the amino acids (aa) constituting the peptide binding structure (the membrane-distal groove) formed by two antiparallel α-helices overlaying an eight-strand β-sheet (Fig. 2B). These variations allow the specific binding of a wide range of 8-11 mers peptides [13]. MHC class I molecules are directly translated, by the ribosome, in the ER lumen. It first encounters two different chaperones: BiP (binding Ig protein) and calnexin (CNX). After the assembly of β_2m, CNX is replaced by its soluble form, calreticulin (CRT) and the complex CRT-MHC/β_2m joins the

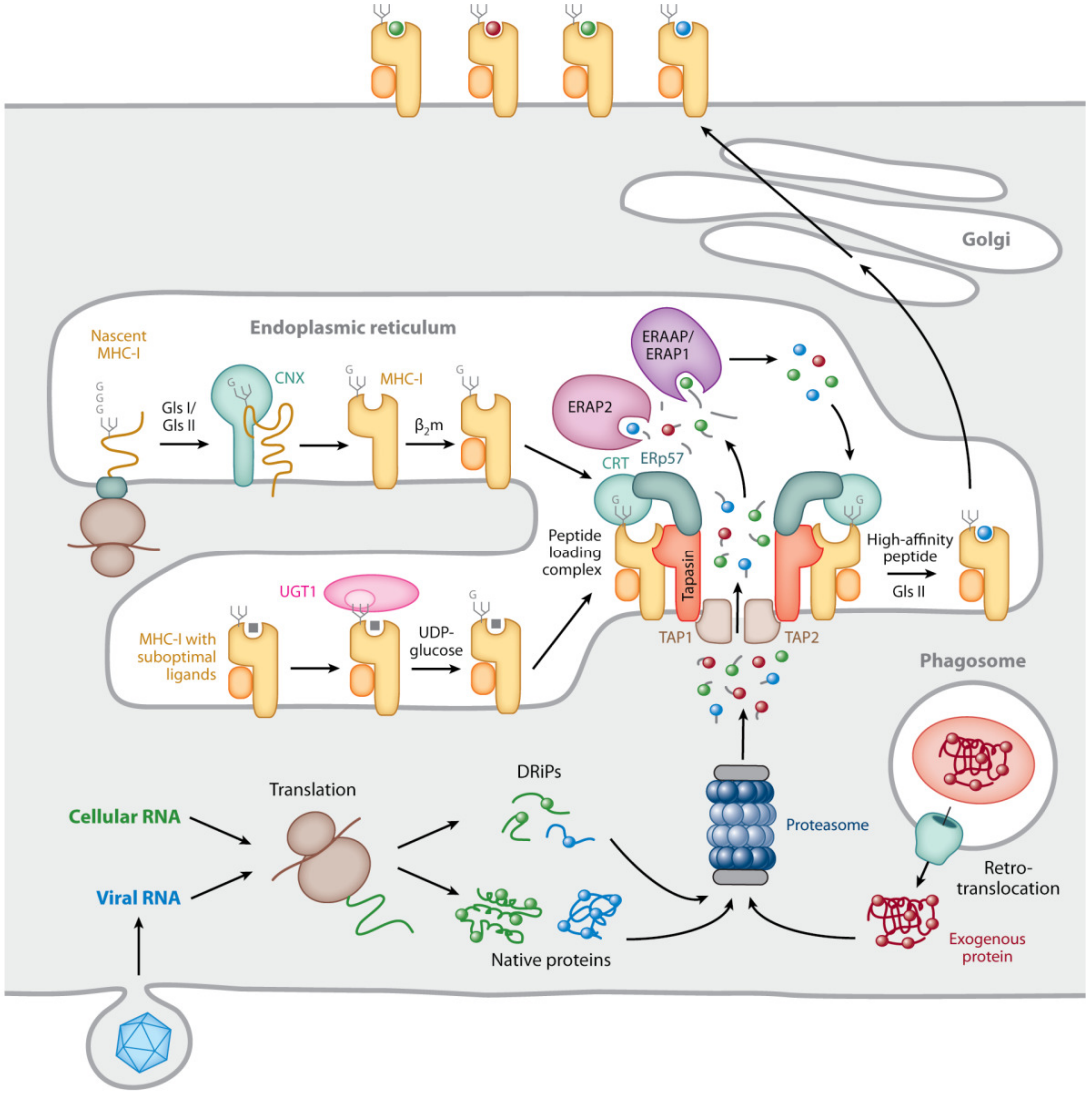

Figure 1: Antigen processing and binding to MHC class I molecules in the Endoplasmic Reticulum (ER).

MHC class I (MHC-I) molecules are synthesized in the Endoplasmic Reticulum (ER), folded by Calnexin (CNX), then assembled with β2-microglobulin (β2m). The MHC-I/β2m complex is then recruited to the Peptide Loading Complex (PLC) by Calreticulin (CRT). In the cell cytoplasm, endogenous proteins and Defective Ribosomal Products (DRiPs), originated from the translation of either self or foreign (i.e., viral) RNA, are degraded into peptides by the proteasome and then translocated into the lumen of the ER by Transporter Associated with Antigen Processing (TAP). These peptides may further undergo trimming by ER aminopeptidases known as ERAAP/ERAP1 and ERAP2 before being loaded on the MHC-I molecules by Tapasin and ERp57. The resulting complex, MHC-I/β2m-peptide is then expressed on the cell surface. Taken from [11], permission number 11163370.

PLC [18] (Fig. 1). This last step allows the stabilisation of the empty MHC class I molecule and the maintenance of its binding groove in a favourable conformation for the high-affinity peptide loading.

In human, MHC class I molecules, or Human Leukocyte Antigen (HLA) class I molecules, can be divided into two groups: HLA class Ia comprising HLA-A, HLA-B, HLA-C, and HLA class Ib, for HLA-E, HLA-F and HLA-G. The same subdivision exists in mice, where the H-2D, H-2K and H-2L subclasses are part of the MHC class Ia molecule group, and H-2Q, H-2M and H-2T subclasses belong to the MHC class Ib, or the non-classical MHC molecules, group.

While MHC class Ia molecules bind to peptides resulting from the degradation of cytosolic proteins by the proteasome, MHC class Ib molecules, specifically HLA-E in human and Qa-1 in mice, bind to peptides derived from the leader sequence of MHC class Ia molecules. Both subclasses' expression depends on the translocation, into the lumen of the ER by TAP, of the specific peptide [19].

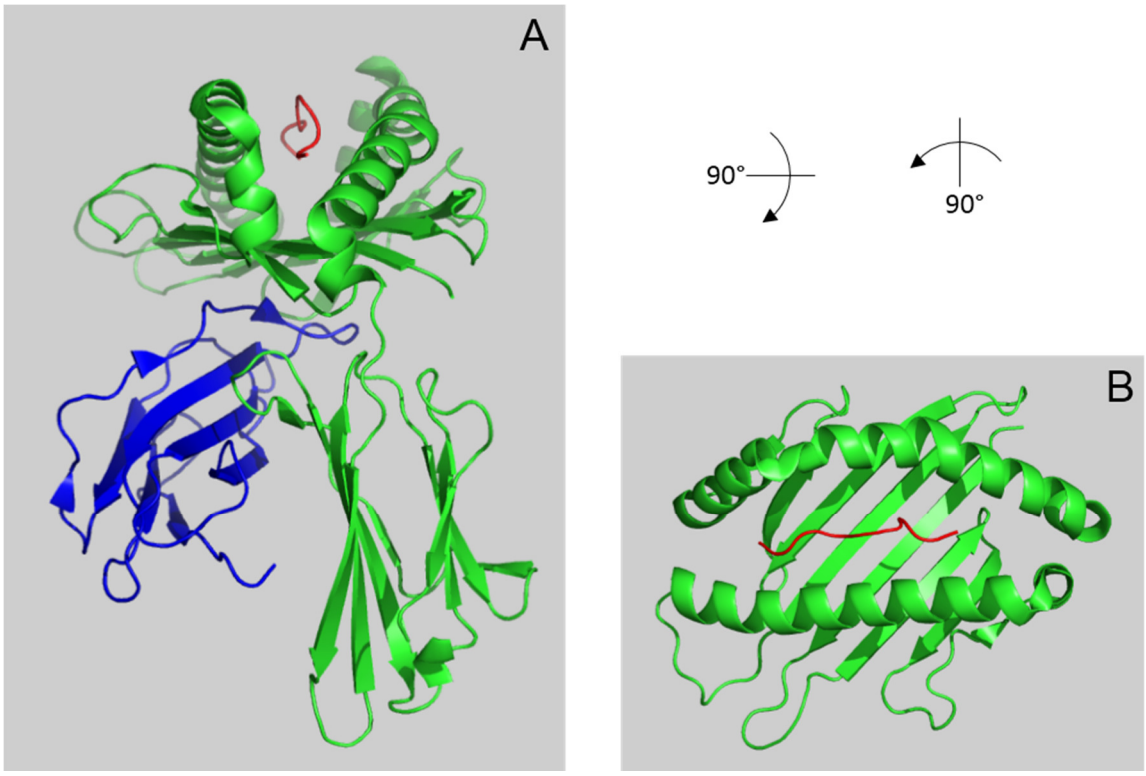

Figure 2: Crystal Structure of a MHC class I molecule complexed with a peptide.

(**A**) Shown is the crystal structure of a MHC class I molecule (HLA-A2) formed by the heavy chain (green) and the β2-microglobulin (β2m) (blue), complexed with a peptide (HCMV pp65-495-503 nonapeptide A7H variant) (red). (**B**) Shown is the membrane-distal groove formed by 2 α-helices and 8 β-sheets (green) presenting the HCMV pp65-495-503 nonapeptide A7H variant (red). PDB file **3MRB**.

1.3. The Proteasome and Protein Degradation

The proteasome, a 26S barrel-shaped structure formed by the assembly of one 20S with two 19S structures, is responsible of the protein degradation in the cytosol. The 20S structure is a four axially stacked rings of seven subunits each ($\alpha_7\beta_7\beta_7\alpha_7$) (Fig. 3). Each of the two outer rings, composed of α-subunits, is capped by a 19S multisubunit complex which recognises, deubiquitinilate then unfold the ubiquitinilated proteins directed for degradation. The resulting proteins are then processed into the centre of the proteasome where the active sites reside. These proteolytic sites are formed by the middle two rings constituted by seven β-subunits, three of which are the active ones: β1, β2, and β5, whose genes (LMP1, LMP2, and MECL1) are MHC-linked [11, 20]. Under interferon-γ (IFN-γ) and interferon-β (IFN-β) induction, these three β-subunits are replaced by other variants (β1i, β2i, and β5i) and form the immunoproteasome [13, 21]. Compared with the proteasomes, immunoproteasomes show a different cleavage pattern resulting in peptides that are more favourable to the MHC class I presentation [13, 22].

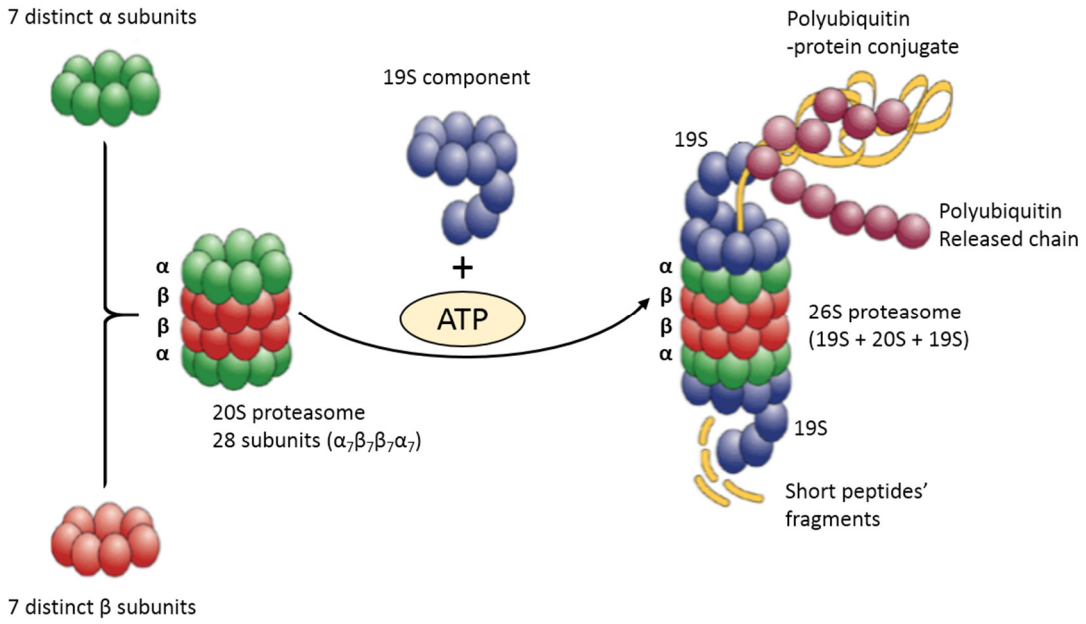

Figure 3: The 26S proteasome structure.

The 26S proteasome is formed by 3 different components, a 20S barrel shaped proteasome caped with two 19S multisubunits. The latter component being essential for the opening of the 20S proteasome as well as the recognition and binding of ubiquitinilated proteins, both functions requiring ATP hydrolysis. The 20S proteasome is an assembly of two inner heptameric rings (β-subunits) containing the catalytic sites, and two outer rings comprising seven different α-subunits. Adapted from [1], permission number 3326560776967.

Peptides presented by MHC class I molecules can have different sources, the major one being endogenous proteins and ones derived from viral genetic material. Another important source of peptides are defective ribosomal products (DRiPs) [23, 24] that include: defective polypeptides resulting from alternative/defective mRNAs, ribosomal frameshift and different translation errors [25, 26]. Other possible sources of MHC class I molecules' binding peptides are specific epitopes generated by nardilysin, thimetoligopeptidase and the insulin-degrading enzyme (IDE) present in the cytoplasm [13].

The products resulting from the proteolysis by the 26S proteasome (20S plus two 19S) are generally 3-22 mers [27], most of them being either too long either too short to be loaded on MHC class I molecules (*cf. chapter 1.2. MHC class I Molecules*). Further trimming, in the cytosol of long peptides is possible by aminopeptidases: leucine aminopeptidase, puromycin-sensitive aminopeptidase, bleomycin hydrolase and the tripeptidyl peptidase II (TPP II) [13, 28, 29]. The role of these aminopeptidases is controversial, especially TPP II that was supposed to be the source of many peptides' generation [30]. These studies where later on contradicted when TPP II knockout (KO) mice were shown to present no major defects in antigen presentation [31, 32]. One clear effect of these aminopeptidases seems to be more negative for the antigen presentation, as they destroy more MHC class I ligands then they create [33].

1.4. TAP protein

1.4.1. History, Structure and Characterisation

Studies on cells with MHC class I expression deficiency revealed deletion of one or several genes in the MHC class II locus region [34, 35]. Mapping of this locus by gene deletion analysis allowed the identification of different candidates involved in the peptide translocation [36]. Four different genes were characterised [37-40] and were later renamed to TAP by the *World Health Organization (WHO) nomenclature committee for factors in the HLA system* [41]: RING4, PSF1, mtp1 and HAM1 are now called TAP1, and RING11, PSF2, mtp2 and HAM2 were renamed TAP2.

TAP belongs to the ATP-binding cassette (ABC) transporter family, which under consumption of adenosine triphosphate (ATP), translocates various substrates across membranes. ABC transporters present a common structure formed by two hydrophilic nucleotide-binding domains (NBD), located in the cytosol, and two hydrophobic transmembrane domains (TMD). The NBD are characterised by highly conserved sequences: Walker A, Walker B, the C, D and Q loops, involved in the ATP binding and/or hydrolysis [4] (Fig. 4A).

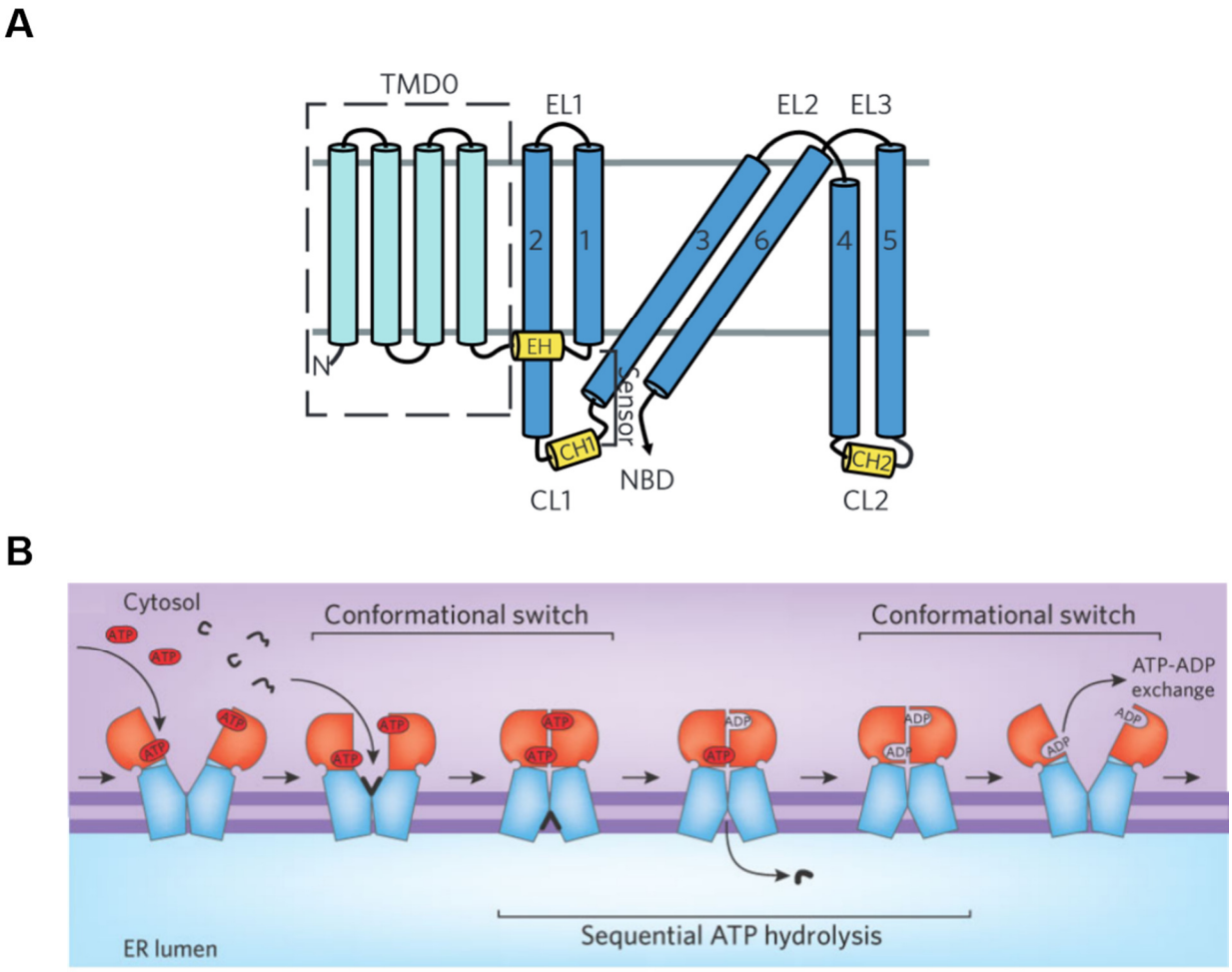

Figure 4: TAP membrane topology, and peptide translocation to the ER lumen.

(**A**) Shown is TAP1 membrane topology (similar to TAP2). EL1, EL2 and EL3 are the ER-luminal loops, CL1 and CL2 the two cytosolic loops, NBD for Nucleotide binding domain, and TMD0 the four transmembrane domains. (**B**) Peptide translocation into the ER lumen requires the hydrolysis of two ATP molecules into two ADP molecules. The cycle starts by TAP loaded with two ATP molecules. Following the peptide binding, a conformational change occurs in TAP leading to the engagement and triggering of the NBDs. The hydrolysis of the two ATP molecules results in the peptide release in the ER lumen and disengagement of the NBDs, restoring the initial TAP conformation. After the ADP exchange to ATP, the cycle starts again. Adapted from [4], permission number 3356010086623.

TAP is a heterodimeric protein composed of 2 subunits: TAP1 (ABCB2) and TAP2 (ABCB3) [42], whose genes are 8 and 12 kb respectively. This protein is anchored in the ER membrane and the *cis golgi*, and transports peptides from the cytosol to the lumen of the ER. No functional homodimers of TAP1 and TAP2 were found [43, 44]: deletions of either or both genes results in a dramatic decrease in MHC class I surface expression which can be corrected by the

addition of exogenous class-I binding peptides [34]. Each of the two TAP subunits can be dissected into: the core TAP complex, or the COOH-terminal NBD, and the NH_2-terminal TMD domain, called TMD_0 which is involved in Tapasin (Tsn) recruitment.

1.4.2. Role of TAP protein in Peptide Translocation

TAP protein translocates peptides into the lumen of the ER by 2 successive steps: the ATP-independent peptide binding [45] followed by its transport into the ER which requires ATP-hydrolysis into adenosine diphosphate (ADP) [46] (Fig. 4B).

The peptide binding to TAP is not a random mechanism as this protein was revealed to be stereospecific [47]: the introduction of D-amino acids leads to a drastic decrease of the peptide binding and translocation. Other peptide characteristics are also important: the peptide's first three N-terminal and the last C-terminal residue are critical for TAP recognition. Such anchor positions allow TAP to bind aa with various lengths (up to 40 residues), as only the two peptide ends are fixed in the TAP binding pocket, the central part being mobile and free of TAP interaction [48, 49].

During the peptide transport, both ATP and the peptide bind TAP independently, leading to two different conformational changes. ATP biding is sensed by the Q loop, inducing the movement of the NBD, resulting in the NBDs dimerization. Structural rearrangement of both TAP1 and TAP2 TMDs is induced by the peptide binding, leading to the peptide release in the ER lumen. Following ATP hydrolysis and the phosphate release, the NBDs dimers are weakened and dissociated. ADP is then released form the complex and replaced by ATP, and the cycle restarts [50].

1.4.3. TAP Protein and Human Diseases

Peptides' presentation by MHC class I molecules is essential for the immune system function. The control of peptide processing and presentation is a key method which enables different pathogens and malignant cells to escape the immune surveillance.

1.4.3.1. Viral Infections

Throughout evolution, different viruses developed ingenious methods to avoid the immune system surveillance by interfering with the antigen processing and presentation and thus, down-regulate MHC class I molecule expression [51, 52]. Till today, 5 different virus-encoded peptides were identified as inhibitors of TAP-mediated peptide transport [52].

The immediate early protein infected cell peptide 47 (ICP47), a Herpes simplex virus

(HSV)-1 and -2 encoded protein, acts as a substrate of TAP and prevents peptide binding [53]. It blocks the essential and first step of the peptide translocation pathway by binding, with high affinity, to the peptide binding site of TAP. This interaction is species-specific and depends essentially on the ICP47 affinity for TAP. Other Herpes viruses, like bovine herpesvirus 1 (BoHV-1), equine herpesvirus 1-4 (EHV-1/4), pseudorabies virus (PRV) also known as Aujeszky's disease, as well as feline Herpesvirus-1 (FeHV-1), encode UL49.5, a protein which interacts with TAP, preventing it from binding to ATP, or targeting it for proteasomal degradation [52].

Human cytomegalovirus (HCMV) encodes gpUS6, an ER-resident glycoprotein. By binding to the ER luminal part of the TAP complex, gpUS6 stabilises the conformation of TAP1 preventing it from binding to ATP [54, 55]. This inhibition can be overridden by IFN-γ-induced TAP overexpression.

Human papilloma virus (HPV) and Epstein-Barr virus (EBV) developed another method to reduce MHC class I surface expression. Once they infect the cell, HPV proteins, as well as the EBV protein vIL-10 (the sequence of which is similar to the human IL-10) down-regulate TAP1 but not TAP2 protein by interfering with its gene expression [56]. Another EBV protein, BNLF2a interacts with the cytosolic domain of TAP1 and inhibits the ATP binding.

E3/19K, an adenovirus protein, interferes with the MHC class I pathways using two different mechanisms: (i) by binding MHC class I molecules and trapping them in the ER lumen, (ii) by binding to TAP protein, preventing its association with MHC class I molecules [57, 58].

Cowpox virus (CPXV) encodes CPXV12, a type II membrane protein, inhibits TAP and down-regulates MHC class I molecule expression [59]. The inhibition mechanism is still to be determined.

1.4.3.2. Tumour Development

Escaping the immune surveillance is the main mechanism of tumour resistance, often ensured by controlling the antigen presentation pathways thus down regulating surface expression of MHC class I molecules [60, 61]. Each of the different antigen processing and presentation steps can be targeted by this regulation, but the most frequent mechanism is TAP malfunction [62]. Some mouse small cell lung carcinoma (CMT.64) and human breast cancer cells showed highly reduced to non-detectable levels of TAP1 or/and TAP2 mRNA [63, 64]. Such a decrease can be restored by IFN-γ treatment, except in some metastatic melanomas where TAP expression increase was not correlated with a higher cytotoxicity [65]. In a study by Johnson *et al*, matched TAP1-negative and TAP1-positive cell lines were generated [66]. Mice inoculated with TAP1-negative cells presented larger and more persistent tumours compared to the TAP1-positive cells. These results were confirmed in a TAP1 deficient mouse lung carcinoma model

(CMT.64) where IFN-γ treatment or infection with a virus encoding TAP1 decreased the tumour growth *in vivo* [67].

1.4.3.3. Transplantation and Autoimmunity Diseases

The role of TAP in autoimmune disease and transplantation is discussed controversially. A varying degree of TAP1 and TAP2 mRNA reduction was observed in many MHC-linked autoimmune diseases, like Sjogren's syndrome, Hashimoto's disease, Graves' disease and others [68]. These observations would presume that such low mRNA levels would reduce the surface expression of HLA class I molecules thus transforming the cells into potential Natural Killer (NK) cell targets.

MHC class I molecules are important players in transplantation as they can induce strong alloreactions directed toward the transplanted tissue. The role of TAP polymorphism in acute cellular rejection is poorly understood. Some TAP2 polymorphisms were found to be correlated with the rejection incidence and severity in the case of renal transplantation [69]. Donors presenting the TAP2*0103 allele were found to be less efficient in presenting allospecific antigens to the recipients T cells. Other studies on patients undergoing liver transplantation associated the increase of early acute cellular rejection to the TAP-1 p.697Gly allele [70]. Further studies in this field are necessary to evaluate the significance of such correlations.

1.4.3.4. Genetic Diseases

Bare lymphocyte syndrome (BLS) is a rare disorder, described for the first time in 1978 [71], and resulting in a reduction of the surface expression of HLA class I, class II, or both classes' molecules (BLS type, I, II or III respectively). Works in 1994 by de la Salle *et al.* [72] have shown that mutations in the TAP1 or TAP2 genes are responsible of the HLA class I defective surface expression in BLS type I patients. Several other patients were later described and grouped under the name of TAP-deficiency (*cf. chapter 2. TAP-deficiency*).

1.5. Peptide Loading Complex (PLC) and Peptide Binding to MHC Class I Molecules

The PLC is a macromolecular machinery responsible of the peptide translocation into the lumen of the ER then its loading on the MHC class I molecule. It is centred on TAP protein and is composed of: the MHC class I heavy chain and β_2m (the peptide presenting molecules), Tsn

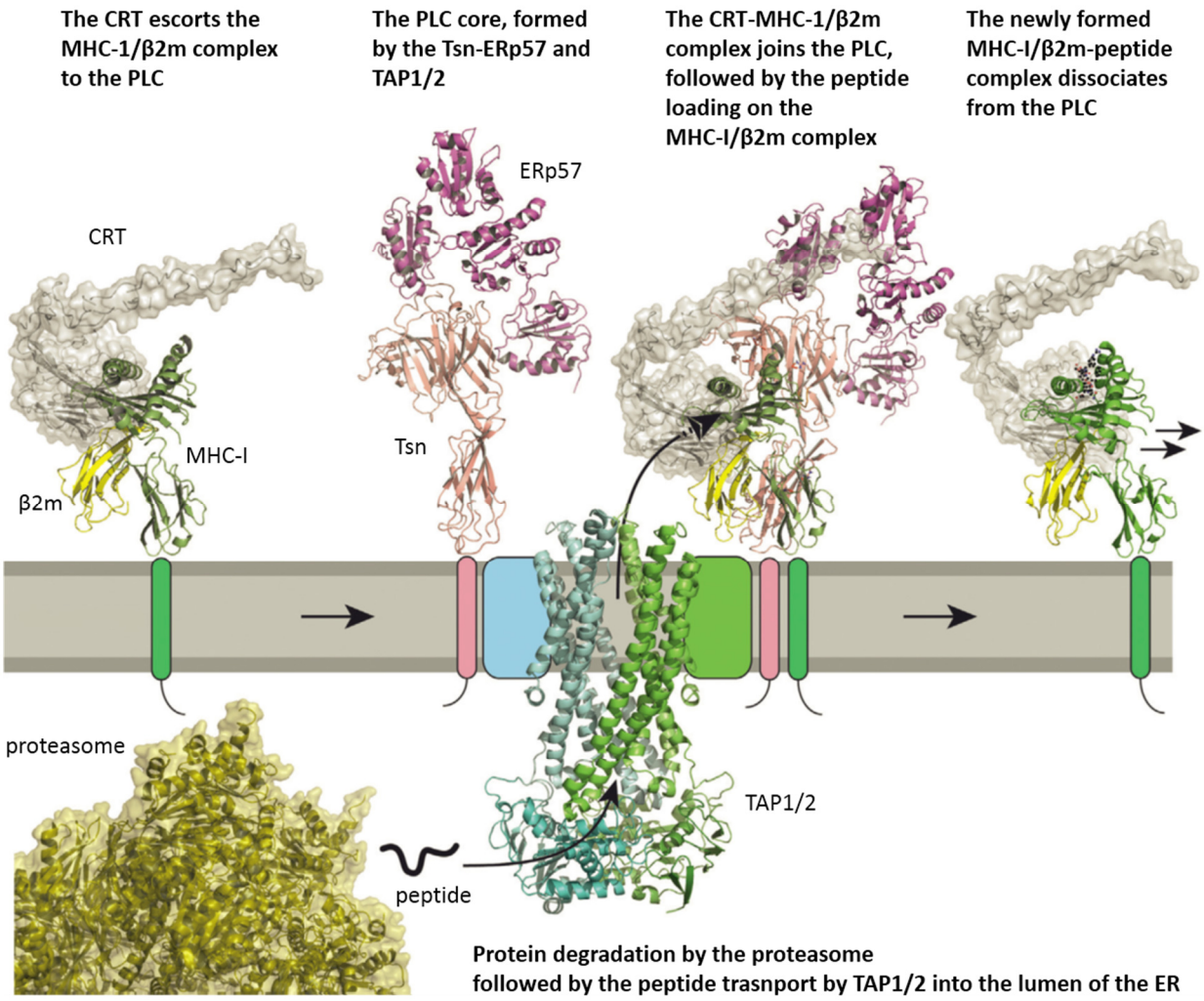

Figure 5: The assembly and disassembly dynamics of the PLC.

The model is assembled based on the crystal structures of HLA-B*4402 (PDB 3KPL), Tsn-ERp57 (PDB 3F8U), CRT (PDB 3RG0), and the homology model of coreTAP1/2 [4]. The different molecules are named in the figure. In the cell cytoplasm, endogenous proteins are degraded by the proteasome into peptides which are then translocated to the ER lumen by TAP1/2. In the ER lumen, the CRT binds to the MHC-I/β2m complex and escorts it to the PLC, composed of the Tsn-ERp57 complex as well as TAP1/2. Once the CRT-MHC-I/β2m complex joins the PLC, the translocated peptides (trimmed by the ER aminopeptidases, not shown) are loaded on the MHC-I/β2m complex and are affinity proofread by Tsn-ERp57. The newly formed, stable MHC-I/β2m-peptide complex dissociates from the PLC, making way for a new, peptide-receptive MHC I to be incorporated into the PLC. MHC I, major histocompatibility complex class I; β2m, beta-2-microglobulin; CRT, calreticulin; Tsn, tapasin; PLC, peptide-loading complex; TAP, transporter associated with antigen processing. Adapted from [13], permission number 3356010828121.

the adapter chaperone, Erp57 the ER-resident oxidoreductase, CRT the lectin-like chaperone and CNX. Both, MHC class Ia and Ib molecule expression is TAP dependant [19, 73, 74]. For optimal peptide loading, the PLC needs to be assembled directly after the peptide translocation to the lumen of the ER by TAP. During the peptide loading process, a sequence of required events have to be fulfilled [13] (Fig. 5).

i. A suitable peptide, for MHC class I loading has to be provided.

   TAP is known to translocate peptides with a wider range of length than the ones optimal for MHC class I loading [73]. Further amino-trimming of these peptides can be performed in the ER by ER aminopeptidase-1 (ERAP1) in human, and ER aminopeptidase associated with antigen processing (ERAAP) in mice [75, 76]. ERAP2, a second aminopeptidase present in humans but not in mice, can play a role in this peptide length optimisation [77]. These aminopeptidases' activity was shown to be crucial, as ERAAP-KO mice produce highly immunogenic and unstable peptides [76].

ii. The TAP/Tsn-ERp57 complex formation.

   An important component of the PLC is Tsn, a type I membrane glycoprotein which plays the role of linker between the MHC class I molecule and TAP. ERp57, an ER-resident oxidoreductase, presents a redox activity which is not essential for the PLC function. Its main and essential role is the PLC complex integrity maintain [78]. In fact, Tsn and ERp57 are covalently linked by a disulfide bond forming a conjugate [79]. This conjugate is then bond to the TMD_0 domain of TAP by the intermediate of Tsn, forming the TAP/Tsn-ERp57 complex which plays a crucial role in the peptide loading. TAP presents two TMD_0 domains allowing the assembly of two different Tsn-ERp57 conjugates.

iii. MHC class I molecule retention in the PLC and the creation of a spatial proximity between TAP and the MHC class I molecules.

   After its formation, the CRT-MHC-I/β_2m complex joins the Tsn-ERp57/TAP complex and form the PLC. In fact, Tsn binds strongly to the monoglycosylated N86 of the MHC class I heavy chain, retaining the latter molecule in the PLC [80]. This step is of high importance as the Tsn-ERp57 complex is responsible for the spatial proximity creation between the peptide donor (TAP) and the peptide acceptor (MHC class I molecule) allowing the peptide binding.

iv. High-affinity binding of peptides to MHC class I molecules.

The main function of MHC class I molecules is to present the peptide turnover on the cell surface for a relatively long time (3-7 days). The stability of this complex is crucial for the immune system function, which explains why MHC class I molecules that are associated with low affinity binding peptides are retained in the PLC. The peptide editing/optimisation is ensured by Tsn, leading to a preferential loading, on the MHC class I molecules, of the high-affinity peptide [81] resulting in a kinetically stable complex.

v. The last step of the peptide loading is the dissociation of the peptide-MHC class I complex from the PLC, a mechanism that is still not completely understood.

2. TAP deficiency

2.1. History and Discovery

In 1978, Touraine *et al.* described the first case of defective surface expression of HLA class I molecules [71]. Similar cases were identified in the following years, and were all grouped under the name of "Bare Lymphocyte Syndrome" (BLS) [82-91]. Depending on the HLA molecule presenting a defective expression, BLS patients were divided into three different types (*cf. chapter 1.4.3.4. Genetic Diseases*). Clinical and immunological parameters allowed the subdivision of BLS type I patients into three distinct groups [72], [92]. (i) Group 1 includes patients presenting the most severe symptoms and are heterozygous for their HLA haplotype, ruling out the possibility that the disease responsible gene(s) are encoded in the HLA locus. (ii) Patients in the group 2 are completely asymptomatic with heterozygous HLA haplotype. The third group is the best characterised and comprises 30 cases (till today). These patients are homozygous for the HLA haplotype and present HLA class I molecule down-regulation caused by mutations in the TAP-1 [93-97], or the TAP-2 gene [72, 98-103].

2.2. Symptoms

TAP-deficient patients express very low levels of HLA class I molecules leading to immune system's malfunction problems. No disease-specific phenotype was found among all the described patients. While some were reported to be asymptomatic [101], others developed symptoms resembling Wegener's granulomatosis syndrome [96, 104]. Nasosinusal involvement and nasal polyposis were among the most frequent symptoms. Based on patients' age, we can distinguish (i) late childhood chronic inflammations of the respiratory tract which could culminate

in respiratory insufficiency resulting from bronchiectasis, emphysema, panbronchiolitis, or bronchial obstruction [92, 105], and/or (ii) adulthood granulomatous skin lesions with no particular pathogen involved [92, 101, 104]. No severe viral infections were reported, suggesting that immune responses by cell-mediated cytotoxicity are, to some extent, efficient [106]. In the opposite, bacterial infections, like with *Staphylococcus aureus, Haemophilus influenza, Escherichia coli, Pseudomonas aeruginosa* etc… are considered to be a real threat for TAP-deficient patients. In some cases, facial lesions eventually lead to complete destruction of the nasal cartilage, and after a generalised vasculitis accompanied with cerebral involvement and seizures, the patient passed away [96, 97, 104].

2.3. Treatments

As mentioned in *chapter 2.2. Symptoms*, no truly specific symptoms exist for this disease, making TAP deficiency diagnosis difficult. The family's medical history and consanguineous marriages may be of great importance during the early diagnosis. Till today, most of the treatments are mainly restricted in the symptoms developments' prevention (rhinosinusitis, bronchiectasis, skin lesions…). In severe cases, bone marrow transplantation was envisaged, but performed only once. The concerned patient seem to recover well, her CD4:CD8 ratio became normal, but her NK cells were still not functional, which is consistent with works, on a mouse model, by D. Raulet group [107]. Such an approach is very risky. Taking in consideration the patient's chronic infections, released cytokines may activate NK cells, leading to a potential cytotoxicity towards autologous cells, already HLA class I low making them a perfect target for activated NK cells [108].

2.4. Effect on the Immune System

Cells derived from TAP-deficient patients express very low surface expression levels of HLA class I molecules (95% to 99% compared to HD). Nevertheless, patients have normal numbers of NK cells, $CD8^+$ α/β T cells exist among their PBMC, and an expansion of γδ T cells was observed in some cases [72, 100]. NK cells derived from TAP-deficient patients were shown to be hyporesponsive against HLA class I negative target cells (no missing-self recognition) [72, 105, 106, 109], which can be reversed after activation with rhIL-2 [106]. Antibody dependent cellular cytotoxicity (ADCC) activity was present for these NK cells, but at lower levels than HD [109], suggesting that TAP-deficient NK cells still have their killing machinery intact. Such data implicate that *in vivo*, TAP-deficient hyporesponsive NK cells may be activated by endogenous interleukins (like IL-2 and IL-15), thus developing a response following viral infections, as

demonstrated in a β2m-KO mouse model [110, 111].

3. NK cells

3.1. Discovery and Characterisation

Till the 1970th, lymphocytes were divided into two main cell types: T cells and B cells. In 1974, then 1975, two independent groups identified a third immune cell type capable of spontaneously killing P-815-Y mastocytomas [112] or the mouse Moloney leukemia cells [113, 114]. This killing, or cytotoxicity, did not require any prior activation nor a host immune sensitisation, so these cells were named Natural Killer (NK) cells. The lymphoid cells' division (B cells, T cells and NK cells) was recently modified, and NK cells are now considered to be part of the innate lymphoid cells (ILCs) subset 1 (ILC1) [115] (Fig. 6).

NK cells constitute 5% - 15% of peripheral blood lymphocytes (PBL) [116] and are present in many other tissues, like bone marrow (BM), thymus, lymph nodes, lung, brain, liver and spleen. In humans, NK cells are defined as $CD3^-$ $CD56^+$ ($CD56^{bright}$ or $CD56^{dim}$), and in C57BL/6 mice as $CD3^-$ $NK1.1^+$ cells. These receptors, and others, will be discussed in *chapter 3.3. NK Cell Receptors*. Although NK cells are known as "killers", other functions were later discovered, like cytokine production and control of the adaptive immunity cells' functions, making NK cells more than just natural killers, but immune cells with a complete and unique role in the organism defence. In this third chapter we will discuss the main aspects of NK cells: their functions, receptors, development and differentiation as well as their education mechanisms.

3.2. NK Cell Functions

NK cells are part of the innate immune system and are involved in early defence mechanisms. They can spontaneously kill stressed, infected and malignant transformed target cells by degranulation or Antibody Dependent Cellular Cytotoxicity (ADCC), produce cytokines, and control adaptive immunity functions. Reverse Antibody Dependent Cellular Cytotoxicity (rADCC) is a specific *in vitro* function of NK cells.

3.2.1. Cytotoxicity

NK cell activities are regulated by a surface detection system composed of a large panel of activating and inhibitory receptors (AR and IR respectively), (*cf. chapter 3.3. NK Cell Receptors*). The balance of the different activating and inhibitory signals transmitted through the

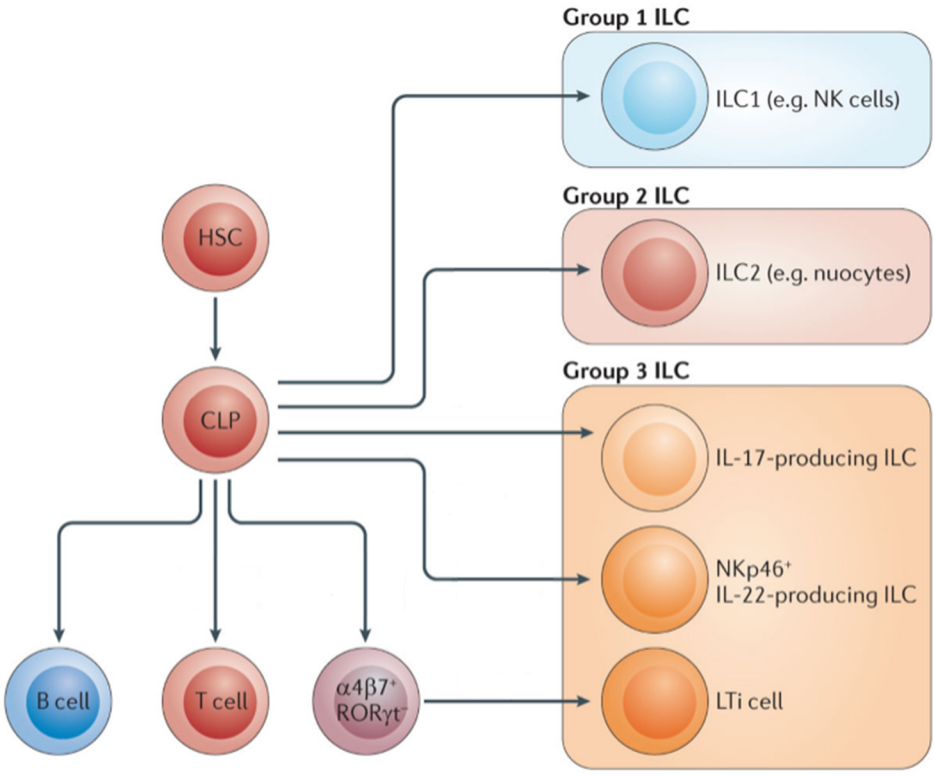

Figure 6: Lymphocytes' subdivision.

Common lymphoid progenitors (CLPs), originated from the haematopoietic stem cell (HSC), give rise to T cells and B cells, as well as innate lymphoid cells (ILCs). The latter cells do not express antigen receptors and can be divided into three major classes: group 1, group 2 and group 3, NK cells being part of ILC group 1. Adapted from [5], permission number 3356480192775.

these receptors determines the target cell's fate [117]. Absence or down-regulation of MHC class I molecules (IR ligands) lead to NK cell activation and target cell killing. This mechanism, called the "missing-self" recognition [118] (*cf. chapter 3.5.1. Missing-self Recognition*), is a result of NK cell cytotoxicity which can be natural (spontaneous) or antibody dependant.

3.2.1.1. Natural Cytotoxicity

Mature NK cells are specialised in target cell killing without any prior stimulation nor antibody recognition [119], and can be mediated through two different mechanisms: perforin/granzyme-mediated cytotoxicity, and the engagement of death receptor pathways [120]. After NK cell activation, both cytotoxicity mechanisms lead, within minutes, to apoptotic cell death by activating caspases, thus limiting the pathogen spread and replication [121].

i. Perforin/granzyme-mediated cytotoxicity.

This mechanism, implicating cytotoxic granules (CG), is the major mechanism of target cell killing by NK cells. CG are specialised secretary lysosomes containing the cytotoxic molecules necessary for target cell killing [122], three of which are: (i) perforin, responsible of target cell membrane disruption [123, 124], (ii) granzymes (Grz) which are related to the serine protease family, responsible of target cell apoptosis triggering by caspase cleavage [121], (iii) and granulysin, a lytic molecule belonging to the saponin-like protein family [125]. NK cell killing by perforin/granzyme-mediated cytotoxicity is evoked by 3 subsequent processes [10, 126] (Fig. 7):

a. Immunological synapse formation.

Once a NK cell encounters a potential target cell, a ligand/receptor interaction occurs leading to the formation of an immune synapse between the two cells. This synapse contains two distinct domains: the peripheral supramolecular activation cluster (pSMAC) and the central supramolecular activation cluster (cSMAC) [127]. The pSMAC containing adhesion molecules (LFA-1 or CD11a/CD18) and rich in actin filaments (F-actin), forms a ring around the cSMAC where the cytotoxic molecules' secretion site is found. This conformation allows an intimate contact between the plasma membranes of both, the effector and the target cell. A former hypothesis has suggested that the cSMAC is an actin free zone, but studies demonstrated that the F-actin is not depleted, it contains openings just wide enough to let the CG pass [128]. The importance of the F-actin formation in this step is demonstrated by the NK cytotoxicity block following cytochalasin D or jasplakinolide treatment, which disrupts or inhibits the actin cytoskeleton rearrangement/polymerisation [129].

b. Microtubule-organising centre (MTOC) polarisation and CG docking to the plasma membrane.

The second step consists of the MTOC and the CG polarisation towards the immune synapse formation site. This step is of great importance as it allows the NK cells to kill specifically the target cell while keeping the surrounding cells unharmed. Cytoskeleton polarisation is induced by the NK AR triggering of Ca^{2+} signalling [130]. In human NK cells, myosinIIA was reported to be essential for the last step of CG release as it promotes their transport through the last layer of F-actin to the site of

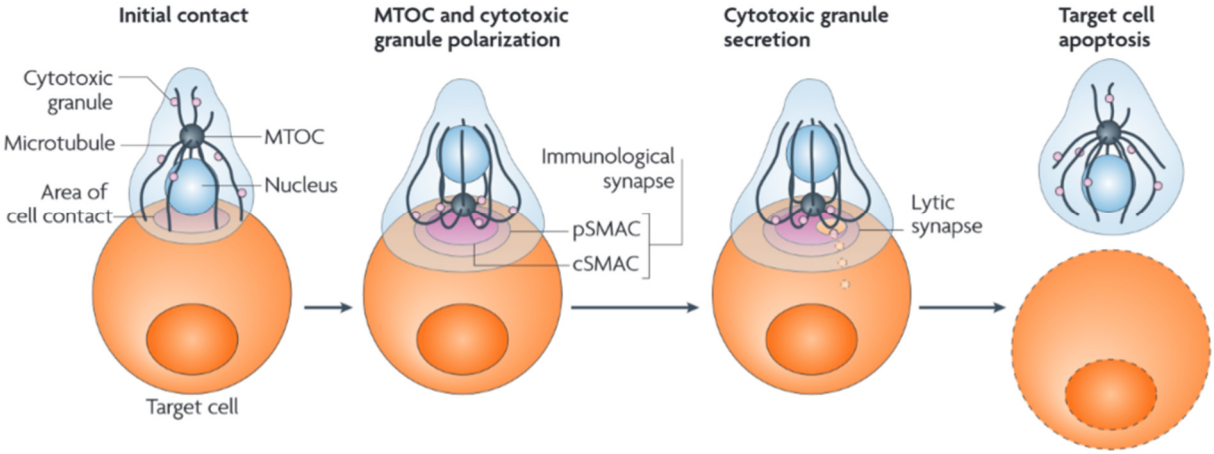

Figure 7: Sequences of events in cytotoxic granules-mediated NK cell cytotoxicity.

Following a ligand/receptor interaction between a NK cell and a potential target cell, an immune synapse is formed. Two different domains can be distinguished in the immune synapse: the peripheral and the central supramolecular activation clusters (pSMAC and cSMAC respectively), allowing an intimate contact between the two cells. Microtubule-organizing centre (MTOC) and the entire microtubule network, as well as the cytotoxic granules (CGs) are polarised towards the immune synapse site. Once the CGs fuse with the NK cell cytosolic membrane, their content (perforin, granzymes…) is released into a small secretory cleft (yellow) formed between the NK cell and target cell, leading to the target cell death by apoptosis. Taken from [10], permission number 3326560924949.

the immune synapse [131, 132]. Once they approach the cytosolic side of the cell membrane, CG dock with the latter membrane, leading to the two membrane fusion and the CG' content release.

c. Cytotoxic molecule release upon fusion of cytosolic and CG' membranes.

In order to release their content, CG have to fuse with the cytosolic membrane, a step catalysed by soluble N-ethyleimide-sensitive factor attachment protein receptors (SNAREs) [133]. SNAREs present a lipid tail allowing them to anchor membranes. Once four different SNAREs, belonging to two different membranes, come together, the result is a fusion of the two corresponding membranes. Cytotoxic molecules are then released in the immune synapse interface leading to the target cell death and the exposure of the CG membrane residing proteins on the cell surface. The most abundant ones are lysosome-associated membrane protein (LAMP) 1 and LAMP2, from which, LAMP1 (CD107a), is widely used as a NK cell degranulation marker [134]. Inactive forms of the cytotoxic molecules are stocked in CG as a result of the low pH and Ca^{2+} concentration. Once they are released in the immune synapse, they become

active and can mediate only target cell killing, leaving the NK cell protected from lysis. Recent works on human NK cells have shown two different and important roles of LAMP1. In fact, this protein is important for lymphocytes' protection from cytotoxic molecules' mediated death [135], and its silencing results in a mobility disturbance of CG leading to an inhibition of NK cell cytotoxicity [136].

ii. Death receptor pathways.

A second mechanism of target cell killing by NK cells involves the activation of the death receptor pathways. These pathways are induced by IFN-γ and result in caspases-dependent apoptosis. Two different pathways will be highlighted in this part: Tumour necrosis factor (TNF) related apoptosis-inducing ligand (TRAIL), and Fas ligand (FasL).

a. TRAIL mediated cell death.

TRAIL, a type II transmembrane protein, belongs to the TNF superfamily. Two of the five TRAIL receptors found in humans, TRAIL-R1 (DR4) and TRAIL-R2 (DR5), are capable of transducing apoptosis signals [137]. In both, humans and mice, TRAIL expression can be induced by IL-2, -15 and type I IFNs. In this cytotoxicity mechanism, IFN-γ play two different roles: TRAIL expression regulation on NK cells, and target cell sensitising to TRAIL-mediated cytotoxicity [138].

b. FasL mediated cell death.

The *in vivo* contribution of the FasL expression by NK cells in tumour growth suppression was demonstrated, even though most of the tumour cells do not express Fas [139]. NK cells, by secreting IFN-γ, induce Fas expression on target cell surface, which needs a direct contact between both cells, demonstrated by double-chamber experiments [140]. FasL is expressed on the inner layer of NK cell CG thus delivered at the same time as perforin to the immune synapse. This may implicate a common and complementary role of both FasL and perforin-mediated cytotoxicity in NK cell target cell killing [141].

3.2.1.2. Antibody Dependent Cellular Cytotoxicity (ADCC)

Upon recognition of antibody-opsonised target cells, NK cell cytotoxicity can be triggered by ADCC [142] which is mediated, in both humans and mice, by the low-affinity IgG receptor, FcγRIII or CD16 [143] (Fig. 8). The resulting triggering signal is high enough to overcome KIR molecule inhibition [144] and leads to NK cell degranulation, cytokine secretion, proliferation, activation marker expression, and apoptosis for some NK cell subsets [145]. Contrary to perforin/granzyme-mediated cytotoxicity, CD16 mediated ADCC induces exocytosis of CG and not their polarisation [146].

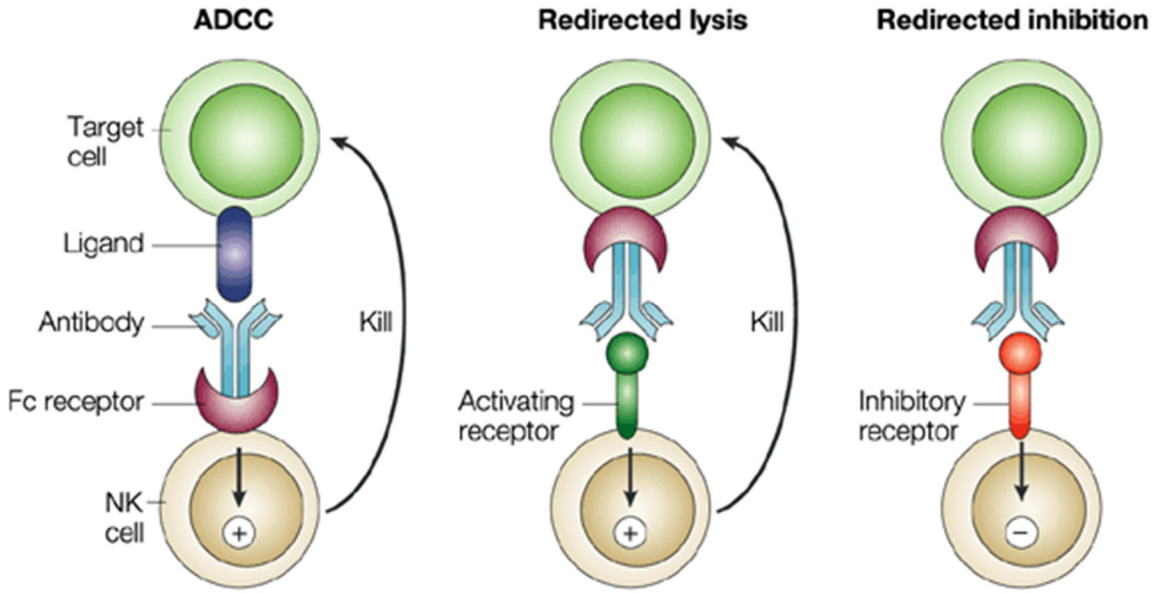

Figure 8: Antibody dependent cellular cytotoxicity (ADCC) and reverse ADCC mechanisms.

Through the recognition of the antibody Fc fragment by their Fc receptor (FcγRIII or CD16), NK cells are able to kill antibody-opsonised cells by antibody dependent cellular cytotoxicity (ADCC). Redirected lysis and redirected inhibition (also known as reverse ADCC or rADCC) are *in vitro* properties of NK cells used to study the effect of specific receptors on NK cell functions. The Fc fragment of a specific NK cell receptor antibody is recognised by the target cell Fc receptor. If the studied receptor is activating, the target cell killing is increased, whereas if the receptor is inhibitory, the target cell killing is reduced. Taken from [2], permission number 3367210630236

3.2.1.3. Reverse Antibody Dependent Cellular Cytotoxicity (rADCC)

rADCC is an *in vitro* NK cell function, never occurring *in vivo* and allows the study of specific receptors' effect on NK cell functions [2] (Fig. 8). The NK cell receptor of interest is recognised, through one of its epitopes, by an antibody. The Fc part of the antibody is then

recognised by the FcγR expressed on the target cell surface. This interaction mimics the one occurring between the studied marker and its receptor on the target cell surface. When implicating a NK AR, rADCC induces the target cell lysis. On the contrary, when the studied marker is a NK IR, the result is a reduction or absence of the target cell lysis.

3.2.2. Cytokine and Chemokine Production

Following their stimulation, NK cells are capable of synthesising and secreting different cytokines which can be implicated in their cytotoxicity functions [147]. These cytokines include IFN-γ, TNF-α, transforming growth factor (TGF)-β, granulocyte-macrophage-colony stimulating factor (GM-CSF), several interleukins as well as the chemokines MIP-1α and MIP-1β [148-150]. During inflammation in a murine model, for example with *Listeria monocytogenes* bacteria, and as a response to high levels of type I IFN or IL-12 and IL-18 secreted by dendritic cells (DCs) and macrophages [151], NK cells are able to secrete high levels of IFN-γ [152, 153], thus activating macrophages and resulting in early *Listeria monocytogenes* clearance [154]. Secretion of cytokines by NK cells also occurs upon target cell recognition and AR triggering and depends on the strength of the stimulation [155] (*cf. chapter 3.3.4.9. Activating Receptors and their Synergistic Combinations*). Cytokine stimulation and target cell recognition can result in a different NK cell response. In human upon cytokine stimulation, the cytokine secretion faculty was found to be higher in $CD56^{bright}$ NK cells compared to the $CD56^{dim}$ subset. The opposite is being true following target cell recognition in which $CD56^{dim}$ NK cells were found to be more potent than the $CD56^{bright}$ subset [155].

As a parallel to T cells which are divided into two sub-types according to their secreted cytokine profile, Th1 (T helper 1) and Th2 (T helper 2), NK cells were also divided into NK1 and NK2 subset regarding their cytokine secretion profile [156]. When NK cells are stimulated with IL-12, a type I cytokine, they produce IFN-γ, another type I cytokine, and they are called NK1. In contrast, NK2 cells secrete type II cytokines like IL-5 and IL-13 upon stimulation with the same type of cytokines, like IL-4.

3.2.3. Adaptive Immunity Control

Alongside their cytotoxicity and cytokine production, NK cells mediate effects on the adaptive immune system by producing a range of immunoregulatory mediators [157-160], as well as interacting with other immune cells. This effect was clearly shown in a NK cell-depleted mouse model where a decrease in the antibody and cytokine response was observed [161]. During early phases of the immune response, a cross-talk between NK cells and antigen presenting cells

(APCs), like DCs and macrophages was suggested [162, 163], leading to both, NK cells' and DCs' or macrophages' activation [164]. Early resistance to bacteria can be mediated by the secretion of IFN-γ, killing of infected cells, and mediating an effect on T regulatory (T_{reg}) response [165, 166], and driving a Th1 polarisation [167]. Upon stimulation with antibody coated target cells, NK cells can produce chemokines which can recruit T cells to the corresponding site [168]. In a murine model, NK cells were found to present a cytotoxic activity against activated $CD4^+$ T cells that do not express sufficient amount of MHC class I molecules. This strategy can be used by blocking the CD94/NKG2A IR, leading to increase of NK cell cytotoxicity against activated $CD4^+$ T cells-dependent autoimmunity [169]. In a mouse solid organ transplantation, graft-resident DCs were found to be eliminated by host NK cells [170].

3.3. NK Cell Receptors

Throughout their developmental process, NK cells acquire, progressively, a mature phenotype and express a large panel of AR and IR, cytokine and chemokine receptors, adhesion molecules (AM) and maturity markers (Fig. 9). These receptors give NK cells the ability to perform their different functions (*cf. chapter 3.2 NK Cell Functions*) and migrate between tissues.

3.3.1. NK Cell Identification Markers

NK cells, originally characterised as ''naturally occurring killer lymphocytes with specificity for tumour cells'' [113, 114], are not defined using a specific surface receptor like T cells and B cells. Since their discovery, many efforts were deployed in finding a specific NK cell marker allowing their identification. NK cells are considered as being non-monocytes, non-B cells, non-T cells, expression CD56 in human, and NK1.1 in C57BL/6 mice. These two receptors are not restricted for NK cells, but are also expressed on subsets of T cells and appear late during NK cell development [171]. One marker, present in basically all species, NKp46, was proposed as a common and specific NK cell marker [172], but the discovery of $NKp46^+$ innate lymphoid cells (ILC) re-launched the search for the ultimate NK cell receptor [173]. Until this specific marker is found, NK cells will still be defined by their surface expression of a certain combination of non-NK-restricted surface antigens; human NK cells will still be defined as $CD3^-$ $CD56^+$ ($CD56^{bright}$ or $CD56^{dim}$), and C57BL/6 mice NK cells as $CD3^-$ $NK1.1^+$ (Fig. 10). CD56 will be developed in the *chapter 3.3.5. Adhesion molecules*, and NK1.1 in the *chapter 3.3.4.6. NK1.1 or NKRP-1C*.

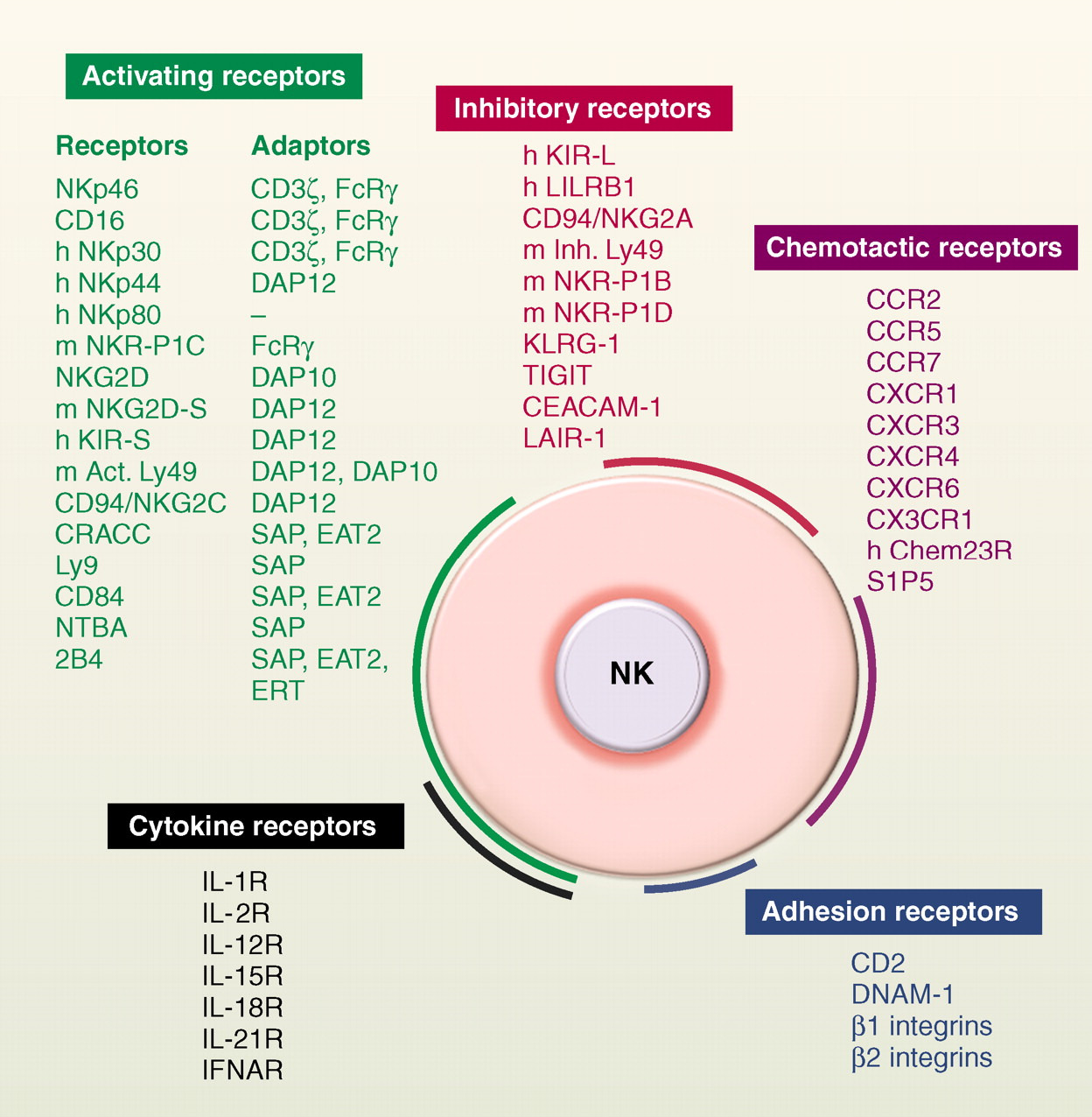

Figure 9: NK cell receptors.

A larger panel of receptors is expressed on NK cell surface and can be mainly grouped in activating (green), inhibitory (red), adhesion (blue), cytokine (black) and chemotactic receptors (purple). Not all NK cell inhibitory receptors are specific for MHC class I molecules. In the activating receptors' list, the adaptor molecules involved in signalling cascades are indicated. Unless indicated (h, human; m, mouse), receptors are conserved in both species. Taken from [7], permission number 3265762809244.

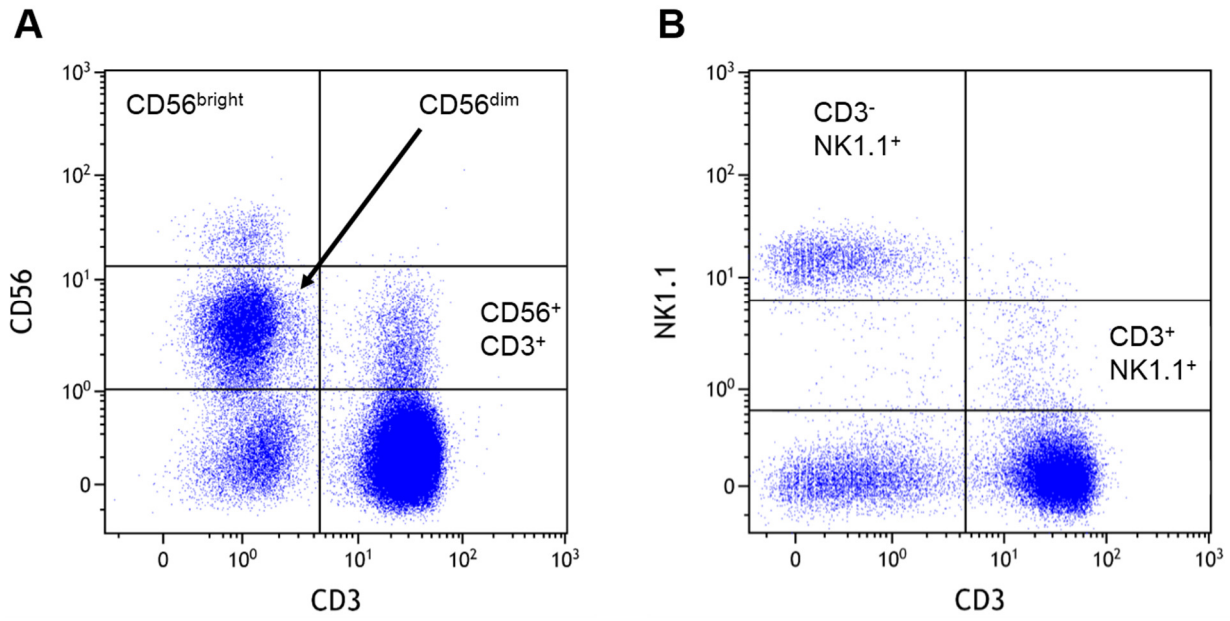

Figure 10: NK cell identification in humans and C57BL/6 mice.

NK cells do not express a specific marker allowing their direct identification. In humans, a combination of CD3 and CD56 is necessary for the identification of two NK cell populations: $CD56^{bright}$ and $CD56^{dim}$ in the $CD3^-$ cells, but also an NKT like population identified as $CD3^+$ $CD56^+$. In C57BL/6 mice, NK1.1 alone is not sufficient for the identification of NK cells, as a $CD3^+$ $NK1.1^+$ population (NKT like cells) needs to be gated out in order to get pure NK cells ($CD3^-$ $NK1.1^+$).

3.3.2. Activating and Inhibitory Signal Transduction

As previously stated, NK cell functions are controlled by a balance between inhibitory signals and activating ones transmitted through a large panel of cell surface AR and IR respectively. These receptors present different transmembrane domains allowing the transduction of their signal to the cell cytosol. Due to their importance in the NK cell function, two different domains as well as their mechanism will be discussed in this paragraph: the immunoreceptor tyrosine-based inhibition motifs (ITIM) and the immunoreceptor tyrosine-based activating motifs (ITAM)

The ITIM motif (consensus sequence S/I/V/LxYxxI/V/L) present in the cytosolic part of most of the immunological IR, is responsible for the inhibitory signal transmission to NK cells in both humans and mice [174, 175] (*cf. chapter 3.3.3. Inhibitory receptors*). IR can contain up to four ITIM motifs. Other immune receptors do not present an ITIM motif, but recruit tyrosine-based motifs for the pathways' effector molecules' activation. AR, in both human and murine species, lack the ITIM motif which is substituted by a positively charged residue (lysine or arginine). This residue, present in the cytosolic tail of the receptor, is required for the interaction with an adaptor

molecule containing an ITAM motif (consensus sequence YxxI/Lx_{6-12}YxxI/L) [176]. Both ITIM and ITAM motifs, upon their surface receptor engagement in an interaction with its specific ligand, can be phosphorylated by SRC (a family of non-receptor tyrosine kinases) leading to other molecules' recruitment and signal transduction.

Once phosphorylated, ITIM motifs recruit phosphotyrosine phosphatases (PTP), like SHP-1 and SHP-2, or SHIP, the inositol-phosphatase. This PTP recruitment leads to a decrease in the phosphorylation of the activation pathway effectors: the spleen tyrosine kinase (SYK), the phospholipase C (PLCγ), the B-cell linker (BLNK/SLP-76) and VAV-1, as well as transmembrane-associated adaptor proteins, like the Fc receptor common gamma chain (FcRγ), CD3ζ (linker for activation of T cells), and cytoskeletal structures. This signalling pathway results in the prevention of calcium signalling and protein kinase C (PKC) activation, thus inhibiting NK cell activation [177, 178].

The phosphorylated sites of ITAM motifs are docked by the tandem SH2 domains of SYK family protein tyrosine kinase (PTK), like the zeta chain-associated protein kinase 70 (ZAP-70). Following this recruitment, a series of substrates, including phosphatidylinositol 3-kinase (PI3K), PLCγ, as well as VAV-2 and VAV-3 are phosphorylated by SYK family PTK. This pathway leads to Ca^{2+} signalling, as well as cellular activation through Ras activation and stimulation of the extracellular signal-regulated kinases (ERK) pathway [179].

3.3.3. Inhibitory Receptors (IR)

All known NK cell IR are part of the inhibitory receptor superfamily (IRS). Most of them carry one or more ITIM motifs in their cytoplasmic tail [174, 175]. The binding of these receptors with their ligand, mostly MHC class I molecules, results in an inhibitory signal delivery allowing the immune cells to spare the target cells. Several ITIM-baring receptors are expressed on NK cells. They include the killer cell immunoglobulin-like receptor (KIR) and immunoglobulin-like transcript (ILT) in humans, Ly49 in mice, and CD94/NKG2 family as well as other receptors in both species. This receptor superfamily can be subdivided into two main groups according to their molecular structure: the immunoglobulin-superfamily inhibitory receptors (ISIR), and the C-type lectin inhibitory receptors (CLIR).

3.3.3.1. Immunoglobulin-Superfamily Inhibitory Receptors (ISIR)

The ISIR family receptors, members of the Ig superfamily type I membrane glycoproteins, include KIR molecules and ILT, both expressed only in humans.

3.3.3.1.1. Killer Immunoglobulin-like Receptors (KIR)

KIR molecules were first discovered in 1995 and designated as “NK-associated transcripts” [180, 181]. This family contains both IR and AR.

i. KIR genes, nomenclature and alleles.

Till today, fourteen genes and two pseudogenes have been identified for KIR molecules, which were mapped to chromosome 19q13.4, the leukocyte receptor complex [182]. These allelic and haplotypic variation are the result of extensive intergenic sequence exchange implicating unequal crossing over, deletions and/or insertions [183, 184]. Their evolution and diversity is highly comparable to MHC class I molecules, their main ligands (especially for the inhibitory KIRs). Each of the genes can encode either an inhibitory or activating KIR, explaining the KIR designation in the literature, for example KIR3DL1/DS1… The subcommittee of the *WHO Nomenclature Committee for Factors of the HLA System* reported the naming rules of the KIR genes which is based on their structure [185] (Fig. 11). After the KIR acronym, a digit indicates their extracellular Ig-like domains number (2 or 3) followed by the letter “D” for Domain, and another letter indicating this domain characteristics which can be Short “S” (presenting activating functions), Long “L” (presenting inhibitory functions) or a Pseudo-gene, “P”. The final digit indicate the gene number. When many genes have the same structure, a “final letter” can be added in order to distinguish them, for example the two genes KIR2DL5A and KIR2DL5B. Like HLA molecules, KIR nomenclature contains information about the genetic characteristics of the different alleles. After their classical name, a set of first three digits indicate the alleles’ differences in the exon region that lead to nonsynonymous changes. The next two digits indicate the alleles’ differences in the exon region that lead to synonymous changes, and the last two digits designate the differences in the alleles’ intron regions, promoter or any other noncoding sequence.

With the rapid evolution of molecular biology techniques, including sequencing, a wider scan of populations’ KIR allele’s polymorphism was possible. The Immuno Polymorphism Database of KIR (IPD-KIR) reported 87 alleles in 2002, 292 alleles in September 2008, then 678 alleles in October 2013, the highest number of polymorphism being for the inhibitory (KIR_DL_) molecules. This high number can be explained by, a correlation between the HLA class I molecules polymorphism and their receptors, KIR molecules’, polymorphism, or simply by the fact that some laboratories have concentrated their effort on studying some specific KIR genes and not others.

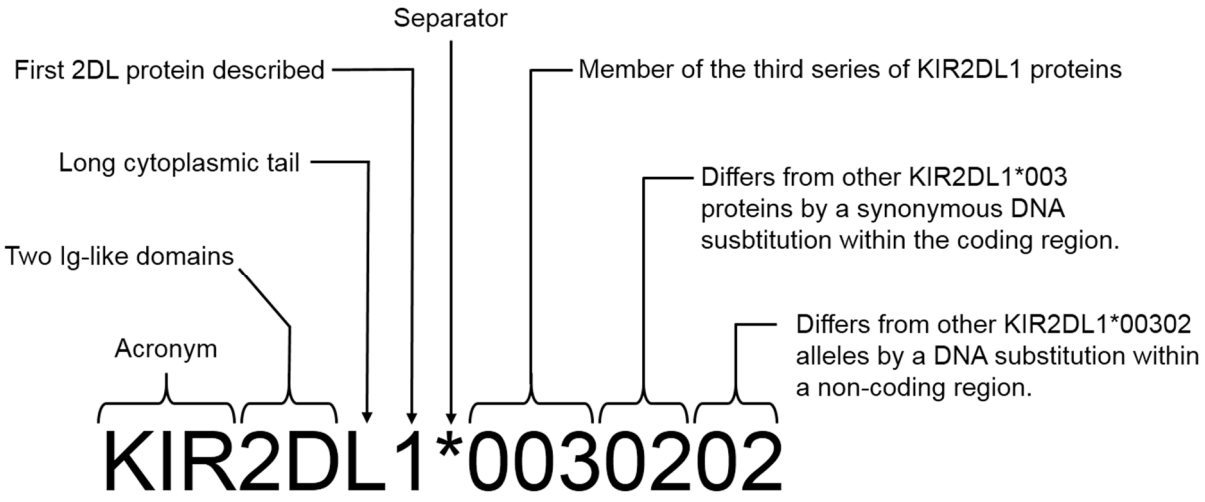

Figure 11: KIR nomenclature.

Shown is the KIR nomenclature rule based on their structure.
Source http://www.ebi.ac.uk/ipd/kir/alleles.html

ii. Haplotypes

Two different KIR haplotypes are determined according to the gene content, namely haplotype A and haplotype B. The KIR region, in the chromosome 19, is divided in two parts: from KIR3DL3 at the centromeric end to KIR3DP1, followed by KIR2DL4 till KIR3DL2 at the telomeric part. These four genes, or framework genes, are present in all humans. The presence of other KIR is used to determine the two known haplotypes. Haplotype A uses eight genes: the four framework genes and four other KIRs (KIR2DL1, KIR2DL3, KIR2DS4 and KIR3DL1), and present mainly allelic polymorphism. The Haplotype B is based on the presence of at least one activating KIR (KIR2DS1/2/3/5, KIR3DS1) as well as KIR2DL5A/B and KIR2DL2. Its variability is mainly determined according to the presence/absence of these genes.

iii. Ligands

HLA class I molecules are the ligands for most of the KIR molecules, especially inhibitory KIRs, with different binding affinities. Inhibitory KIR molecules present a long cytosolic domain with one or more ITIM motifs. Some HLA class I residues favour certain interactions to others. HLA-Cw alleles are present in all humans, making it the most important regulator of NK cell functions. HLA-C molecules can be divided in two different groups. HLA-C group 1, which includes HLA-Cw1, 3, 7 and 8, can bind KIR2DL2 and KIR3DL2 using the Asp (asparagine) at position 80, whereas HLA-C group 2, which

includes HLA-Cw2, 4, 5 and 6, uses the Lys (lysine) 80 to bind KIR2DL1 [186]. Whereas the KIR2DL1 interaction with the Lys80 of HLA-C2 group is very specific, one report showed that KIR2DL2, and to a weaker extend KIR3DL2 can bind HLA-C2 group [187]. Other KIR molecules, like KIR3DL1 specifically bind the Ile/Thr80 of HLA-Bw4 and HLA-A molecules [188]. KIR3DL2 selectively bind HLA-A3 and -A11 alleles only when they are loaded with specific viral peptides [189]. Finally, KIR2DL4 is the specific receptor for HLA-G [190].

3.3.3.1.2. Immunoglobulin-like Transcripts (ILT)

Immunoglobulin-like transcripts (ILT), or leukocyte immunoglobulin-like receptors (LIR), are genetically related to KIR molecules [183]. They both possess isoforms with different cytosolic signalling functions. ILT/LIR receptors with long cytoplasmic tails recruit SHP-1 phosphatase to the ITIM motifs thus inhibiting cellular activation [175]. The ILT/LIR family includes several receptors, like ILT2 (LIR-1), ILT3 (LIR-5), ILT4 (LIR-2), ILT5 (LIR-3) and LIR-8 [22, 191].

ILT2, LIR-1 or CD85j presents four extracellular Ig domains and four cytoplasmic ITIM motifs [192]. It is expressed on basically all PBMC and is able to inhibit the Ca^{2+} flux in B cells, monocytes, macrophages and DCs [193]. T cell and NK cell cytotoxicity is also inhibited by ILT2 [193, 194]. ILT2/LIR-1 binds a wide range of HLA class I molecules: HLA-A1, -A2, -A3, HLA-Bw4 and -Bw6, HLA-Cw3 and -Cw4 as well as HLA-G [194]. It can also bind molecules with a similar structure to HLA class I molecules, like the CMV protein UL18 [195]. However, ILT2 does not seem to play a role in NK cell education, and more evidence is needed in order to demonstrate its real role in NK inhibition *in vivo* [196].

Other ILT/LIR family members were detected only as transcripts in NK cells, no surface expression was found, like ILT3 (LIR-5), ILT4 (LIR-2) and LIR-8 [191]. Their expression on other PBMC allowed the identification of the ILT3 ligand only, being HLA class I molecules, with the same wide range of binding as ILT2/LIR-1 [197].

3.3.3.2. C-type Lectin Inhibitory Receptors (CLIR)

The CLIR family are members of the lectin C superfamily, type II membrane glycoproteins. Some are specific for mice, like Ly49 family and NKR-P receptors, whereas others are common for both humans and mice, like the inhibitory CD94/NKG2 family, and KLRG1.

3.3.3.2.1. Inhibitory CD94/NKG2

The NKG2 gene complex, located on the chromosome 12p13.2 in humans and chromosome 6 in mice, presents inhibitory and activating receptors. Both inhibitory and activating forms are represented by a disulfide-linked heterodimer formed by two molecules, CD94 and NKG2 [198]. (for CD94, *cf. chapter 3.3.6. Other NK Cell Phenotypic markers*). The NKG2 receptors are always expressed on the cell surface as CD94/NKG2 complex. This complex, in its inhibitory as well as activating form, recognise the non-classical MHC class I molecule, HLA-E in humans [199], and Qa-1 in mice [200]. In humans, the two inhibitory forms of this complex are CD94/NKG2A and CD94/NKG2B, NKG2B being a splicing variant of NKG2A missing 18 aa between the transmembrane domain and the carbohydrate recognition domain [201]. CD94/NKG2A and CD94/NKG2B recognise HLA-E or Qa-1 molecule loaded with specific peptides, and transduces an inhibitory signal through its one or two cytosolic ITIM motifs, in mice and human respectively [19, 199, 200]. Both CD94 and NKG2A chains are implicated in the interaction with HLA-E: while NKG2A interacts with the α2-helix of HLA-E, the α1-helix of the latter molecule and the C-terminal part of the peptide are oriented toward CD94. This last interaction, CD94-peptide, is important for inducting the inhibitory signal [202-204].

3.3.3.2.2. Killer Cell Lectin-like Receptor G1 (KLRG1)

KLRG1 is a type II transmembrane molecule present in humans and mice, and is preferentially expressed by NK cells and antigen-experienced T cells [205, 206]. It binds the highly conserved region of E-, N- and R-cadherins [207] and acts as an IR. The cytosol tail of KLRG1 contains an ITIM motif which recruits the phosphatases SHP-2 and SHIP-1 conferring its inhibitory functions [208, 209]. Different infections (bacterial, viral and parasitic) result in an expression of KLRG1 on NK cells and T cells [210, 211]. An increase of KLRG1 expression levels was observed on human NK cells after chronic HCV infection as well as NK cells co-cultured with hepatocytes expressing HCV. [212].

3.3.3.2.3. Inhibitory Ly49

Unlike humans, mice do not present KIR and ILT/LIR molecules, but instead they express proteins belonging to the type II glycoproteins, the Ly49 family. Ly49 proteins are expressed on the cell surface as disulfide-linked homodimers [213]. They recognises MHC class I molecules, the H-2 molecules, and can be activating or inhibitory depending on the intracellular domain they express or interact with (ITAM or ITIM respectively). Although human KIR molecules and murine

Ly49 receptor family have analogous functions and recognise MHC class I molecules, they are both structurally distinct. The difference between these two molecules' families is that big that they cannot have shared a common evolutionary descent [188].

The Ly49 family includes up to 30 different genes encoding inhibitory, activating, and many pseudogenes. Based on homology, genomic organisation, and function, groups of Ly49 genes can be clustered as shown in Fig. 12 [214]. Ly49A, or T lymphocyte activation marker, was the first one to be described [215]. Not all murine strains share the same set of Ly49 receptors, and when they do, the same receptor may present: allelic variants depending on the strain, like $Ly49A^{B6}$ and $Ly49A^{Balb/C}$, specific for the C57BL/6 and Balb/C strain respectively, or homologues in the different strains, like $Ly49E^{Balb/C}$ and $Ly49E^{NOD}$ present in the Balb/C and the Non-obese Diabetic (NOD) strain respectively.

Most of the Ly49 proteins, especially the inhibitory ones, recognise the different MHC class I molecules, H2-D, H2-L and H2-K, with different affinity, the latter three molecules being specific for each of the murine strain [214, 216, 217]. For example, Ly49A can bind to the two MHC class I molecules present in C57BL/6 mice, $H2\text{-}K^b$ and $H2\text{-}D^b$, but it can also recognise, with higher affinity, the $H2\text{-}D^d$ and $H2\text{-}L^d$ molecules expressed in Balb/C mice [218]. This interaction can occur in *cis*, when both, the receptor and the ligand, are expressed on the same cell surface, or in *trans*, when each is expressed on a different cell. These two different interactions involve molecular shifts in the receptor and ligand conformation leading to different signal transduction [219], developed in the *chapter 3.5.5. Cis and Trans Interactions*.

3.3.4. Activating Receptors

NK cell killing does not occur just in the absence of inhibition, but next to that, it requires activating signals which are generally transmitted through a panel of surface AR associated with adaptor proteins carrying ITAM motifs. AR belong to many different receptor superfamilies, but most of them, like IR, belong to the Ig and the C type lectin families. Many of the IR have activating counterpart, like KIR, ILT/LIR, CD94/NKG2 and Ly49. AR can be autonomous, capable of stimulating NK cells upon their unique engagement with their ligands, or co-stimulatory, when a second stimulus is needed to ensure their activating signal transduction. They have a much wider range of ligands compared to IR as they can bind constitutive cellular molecules as well as stress-induced proteins.

A

A group(inhibitory) a^{B6}, a^{NOD}, a^{Balb} , o^{129}
B group(inhibitory) b^{129}, b^{B6}, $b^{Balb/c}$
C group(inhibitory) $i_{1-2}{}^{NOD}$, $i_1{}^{129}$, i^{B6}, i^{Balb}, c^{NOD}, $c^{Balb/c}$, c^{B6}
E group(inhibitory) e^{NOD}, e^{B6}, e^{129}, e^{Balb}, f^{NOD}, f^{B6}, s^{129**}
G group(inhibitory) t^{129}, $g_{1-2}{}^{NOD}$, g^{B6}, g^{129}, g^{Balb}
Q group(inhibitory) $q_1{}^{129}$, q^{NOD}, q^{B6}, $q^{Balb/c}$

D group(activating) d^{B6}, d^{NOD}, $p_{1-3}{}^{NOD}$, p^{129}, pd^{129}, $pd_{1-2}{}^{NOD}$, x^{B6}, x^{NOD}, r^{129}
H group(activating) n^{B6}, k^{B6}, h^{B6}, h^{NOD}, u^{NOD}, u^{129}
L group(activating) l^{Balb}, w^{NOD}, m^{B6}, m^{NOD}

B

Strain	Gene number
Babl/c	8
C57BL/6 (B6)	15
129S6	19
NOD	21

Figure 12: Ly49 genes' clusters and mouse strain expression.

(**A**) Ly49 genes can be subdivided into different clusters. After each gene name, the corresponding mouse strain is indicated as a superscript. Blue is used to indicate inhibitory Ly49 genes, and red for activating Ly49 genes. Ly49 gene number differs between strains as shown in (**B**). Adapted from [3].

3.3.4.1. Natural Cytotoxicity Receptors (NCR)

NCRs belong to the Ig superfamily type I membrane proteins [220]. Structurally, they are composed of three different domains: an extracellular binding-domain containing the membrane distal and the membrane proximal, the latter being important for ligand binding, a transmembrane domain associated with an adaptor molecule (possessing an ITAM motif), and a short cytosolic domain [154, 221, 222]. Cellular heparin, or heparin sulphate proteoglycans, upregulated on cancer cells, are a common ligand for all the NCRs [223]. NCRs can also recognise pathogen associated ligands [224]. An overview of three different NCRs, and their ligands will be presented below.

i. CD337 (NKp30 or NCR3)

CD337, expressed only in humans, is associated with an adaptor protein bearing an ITAM motif, such as homodimers of CD3ζ chains linked with disulfide bonds, or CD3ζ and γ-chain of the high-affinity Fc receptor for IgE (FcεRI) [225, 226]. Three different isoforms

exist for NKp30: NKp30a and NKp30b, both stimulatory, and NKp30c which is immunosuppressive [227]. NKp30-mediated NK cell killing results of its binding to one of two different surface proteins: B7-H6, which was exclusively found on tumour cell surface [228], and BCL-2-associated athanigene 6 (BAG6 or BAT3), found on immature DCs surface [229, 230]. NKp30 can also bind the HCMV protein pp65 which inhibits NKp30-mediated NK cell killing by dissociating the AR from its adaptor molecule, CD3ζ [231].

ii. CD336 (NKp44 or NCR2)

NKp44, encoded by the human NKC2 gene, is only detected on activated NK cells [232]. It has a cytosolic ITIM motif, which does not seem to be functional, and its signal transduction is mediated by the ITAM-containing adapter DNAX-activation protein (DAP)12 [233]. Many ligands were described for NKp44, including influenza virus HA and other viral HA-neuraminidase (HN) proteins [221, 234], sialylated and sulphated cellular proteoglycans [235], and bacterial wall elements [236]. Proliferating cell nuclear antigen (PCNA) is an intracellular protein, but is sometimes recruited on tumour cell surface where it is recognised by NKp44. This protein acts as an inhibitory receptor for NKp44 allowing the tumours to escape NK cell killing [237]. NKp44-mediated killing of noninfected $CD4^+$ T cells was reported. In fact, after an aborted viral fusion, the conserved 3S motif (SWSNKS) of the HIV-1 gp41 envelope protein, binds to the complement receptor gC1qR on $CD4^+$ T cells, which is then recognised by NKp44 [238, 239]. Recently, a new NKp44L has been found, an isoform of mixed-lineage leukemia-5 (MLL5), not detectable in normal tissues, but present on a broad range of tumour and transformed cells [240].

iii. CD335 (NKp46 or NCR1)

NKp46, the only NCR expressed in humans and mice, was long thought to be the specific NK cell marker, but a subset of ILCs expressing NKp46 was discovered [241]. Its short cytosolic tail is associated with CD3ζ and the FcεRI γ-chain [233]. NKp46 ligands are unknown to date, but are expressed on many cell types, like tumour cells [242, 243], pancreatic β-cells, inducing type I diabetes [244], and liver stellate cells, protecting from liver fibrosis [245]. Some viral Hemagglutinin (HA) (form influenza and Sendai virus) were found to be NKp46 ligands [222]. Resting NK cells stimulation with NKp46 is not sufficient to induce degranulation. Combination of NKp46 signal with other receptors' signals (CD2, CD244, DNAM-1 or NKG2D) was necessary for NK cell activation [246]. Among the three NCRs, only NKp46 functions can be studied *in vivo*. Two different NKp46 mutants were generated: the $Ncr1^{gfp/gfp}$, in which the exons 5-7 were replaced by eGFP [234], and the

Ncr1$^{Noé/Noé}$, presenting a substitution of the residue 32 to arginine [247]. Ncr1$^{gfp/gfp}$ mice developed tumour metastasis and were vulnerable to influenza virus infections [248, 249], whereas NK cells from Ncr1$^{Noé/Noé}$ mice presented NKp46 receptor-independent hyper-responsiveness [247]. The intact NKp46 DNA, RNA and protein are present in Ncr1$^{Noé/Noé}$ mice, but lack in Ncr1$^{gfp/gfp}$ mice. This major difference may explain the discordance in these two models' responses. Whatever the differences between these two models are, they both demonstrate an important role of NKp46 in the recognition/elimination of tumours, viruses and bacteria.

3.3.4.2. Activating CD94/NKG2

The activating form of the CD94/NKG2 complex consists of two different molecules: CD94/NKG2C and CD94/NKG2E which exist in human and mice. The activating CD94/NKG2 adaptor molecule is DAP12 which presents two ITAM motifs that transduce the activating signal. Both CD94/NKG2C and CD94/NKG2E, like their murine inhibitory counterparts CD94/NKG2A and CD94/NKG2B, bind Qa-1. In human, CD94/NKG2E ligand is still unknown, whereas CD94/NKG2C binds HLA-E molecule with 6 fold weaker affinity than CD94/NKG2A and CD94/NKG2B [250]. This might be due to the difference in the aa residues 167-170 of the NKG2/CD94 interface [250, 251]. In the opposite, the HLA-E molecule expressing the signal peptide of HLA-G is equally recognised by both inhibitory and activating CD94/NKG2 complexes [252]. The HCMV protein, gpUL18, is a MHC homologue which can be recognised by CD94/NKG2C with a weak affinity [251]. gpUL18 can also bind ILT2/LIR-1 (*cf. chapter 3.3.3.1.2. Immunoglobulin-like Transcript (ILT)*). Both interactions are opposed as the gpUL18 interaction with CD94/NKG2C leads to NK activation, whereas its interaction with ILT2/LIR-1 inhibits NK cell functions [195, 253]. In the CD94/NKG2 family, other AR exist, like CD94/NKG2F and CD947NKG2I, but they will not be developed in the thesis introduction.

3.3.4.3. NKG2D or CD314

NKG2D is one of the most studied NK cell AR, and its activating role is largely demonstrated. Contrary to what its name may suggest, NKG2D is different from the NKG2 family. It is expressed as a homodimer, not associated with CD94 molecule [254]. In humans, NKG2D binds MICA and MICB, expressed upon cellular stress and in some epithelial tumours. MICA and MICB are structurally similar to MHC class I molecules, but do not associate with β2m molecules nor bind peptides [255]. UL16-binding proteins (ULBP) or retinoic acid early transcripts 1 (RAET1)

are also ligands for NKG2D [256, 257]. UL-16 is a CMV protein which binds to ULBP1 and ULBP2 as well as MICB and retain them in the ER, preventing their recognition by NKG2D, thus inhibiting cell lysis by NK cells. In normal conditions, ULBP transcripts are generally present in many cell types, but not expressed on the cell surface [256]. This expression is restricted to many tumours and infected cells [254, 257]. In mice, NKG2D recognises, the family of Rae I (homologue of the human RAET1), as well as the histocompatibility 60 protein (H-60), similar to the MHC class I molecules, and the murine ULPB-like transcript 1 (MULT1) molecules [254].

In human, the NKG2D adaptor molecule is DAP10 which carries a tyrosine based motif (YxxM) different than ITAM motifs [258], and recruits the phophatidylinositol-3 kinase (PI3K) resulting in an activation pathway leading to CG polarisation toward the target cell and degranulation [259]. Two different isoforms of NKG2D were found in murine NK cells. The difference is based on their cytosolic tail length: NKG2D-L having a Long cytosolic tail, associated with DAP10 and expressed on both resting and activated NK cells, and NKG2D-S, presenting a Short cytosolic tail, an isoform only expressed on activated NK cells and associated with DAP10 or DAP12 adaptor molecules [260].

3.3.4.4. Activating KIR and LILR/ILT/LIR

Activating and inhibitory KIR and LILR/ILT/LIR receptors exhibit high degrees of sequence identity especially in their extracellular part suggesting a comparable ligand specificity. Activating KIR interaction with their ligands seems to be very weak making these latter molecules' identification very difficult. Some activating KIR ligands are still unknown, like those of KIR2DS3, 2DS5 and 3DS1. KIR2DS1 and 2DS2 were found to bind to the same ligands as their inhibitory counterpart (KIR2DL1 and DL2), HLA-Cw4, but with a much weaker affinity [261]. KIR2DS4, using soluble Ig-Fc molecules, recognises HLA-Cw4 (but not –Cw6) molecules [262]. Activating KIR and LILR/ILT/LIR receptors present short cytoplasmic tails. Their transmembrane region has a charged residue associated with signalling adaptor proteins possessing ITAM motifs (TYROBP/DAP-12) which is responsible of the activation signal transmission [263]. An exception for this rule is KIR2DL4, with soluble HLA-G as ligand, presents atypical domains. Although its two extracellular Ig domains are connected to an intracellular ITIM motif, KIR2DL4 exhibit activating functions mediated by a positively charged aa (arginine), located in the amino-terminal part of the transmembrane domain) and connected to an adaptor molecule, FcεRIγ.

3.3.4.5. Activating Ly49

Like the human KIR and ILT/LIR molecules, murine Ly49 proteins can also be activating

receptors. Both activating and inhibitory forms are highly similar in their extracellular portion especially their ligand recognition domain [213]. Unlike the inhibitory Ly49 proteins (which are expressed on NK cells, NKT cells and a part of T cells), the activating Ly49 proteins are restricted to NK cells [264]. It was demonstrated that the transgenic expression of Ly49D and its interaction with its ligand (H2-D^d, H2-D^r and H2-D^{sp2}) are incompatible with NKT cells' development [265]. Act Ly49 protein are coupled to the adapter molecules DAP12 and DAP10 [266] and are implicated in direct recognition, on infected target cells, of viral proteins resembling MHC class I molecules [267]. A good example of the act Ly49 molecules in the NK cell-mediated antiviral defence is Ly49H, which recognises the CMV protein m157, structurally close to the MHC class I molecules. This interaction, Ly49H-m157, induces NK cell cytotoxicity and IFN-γ secretion [268]. Following CMV infection, a preferential expansion of $Ly49H^+$ NK cells is detected, which stops once the adaptive immune response appears [269]. Till today, no MHC class I capable of interacting with Ly49H was found, indicating that this AR is specific of a virus-encoded protein [270].

3.3.4.6. NKRP-1C or NK1.1

NK1.1, CD161c or NKRP-1C belongs to the type II C-lectin superfamily and does not have an ITIM nor an ITAM motif in its cytosolic tail. It is expressed as a homodimer on the surface of C57BL/6 murine NK cells and a small subset of T cells [271]. NK1.1 is used as C57BL/6 identification marker. Unlike $TCR\alpha/\beta^+$ cells, $NK1.1^+$ T cells are able, like NK cells, to spontaneously lyse several kinds of tumour cells in vitro [272, 273]. The inoculation of anti-NK1.1 antibodies to C57BL/6 mice results in NK cells death by apoptosis, but $NK1.1^+$ T cells proliferation and IL-4 secretion [274], whereas in rADCC experiments, the anti-NK1.1 antibody stimulates NK cells for cytotoxicity and IFN-γ secretion [275, 276].

3.3.4.7. CD16 and CD32

CD16 or FcγRIII, expressed in both human and mice, is part of the Ig superfamily, presents two extracellular Ig domains, a transmembrane charged aa and a short cytosolic tail that does not have an ITIM nor an ITAM motif [277]. It presents two different isoforms: CD16a, expressed on NK cells and co-localising with homodimers or heterodimers of CD3ζ and Fc-εRI-γ in human, and only with Fc-εRI-γ homodimers in mice, and CD16b, expressed on neutrophils [278, 279]. CD16a is one of the most important NK cell AR as it induces ADCC (*cf. chapter 3.2.1.2. Antibody Dependant Cellular Cytotoxicity (ADCC)*) by recognising the Fc fragments of IgG1 and IgG3 but not IgG2 nor IgG4 and inducing NK cell activation [143]. It has been shown that after human NK

cell co-culture with malignant target cells, a down-regulation of CD16a occurs, which might be important for NK cell function modulation [280, 281].

NK cell ADCC can also be induced by another Fc receptor, FcγRIIc1, an isoform of CD32 which recognises Fc parts of IgG3, IgG1 and IgG4 as well as IgG2 but with a much weaker affinity. This AR is a part of the Ig superfamily, like CD16, but differs in the fact that it presents a cytosolic ITAM motif responsible of its activating signals transduction. Two different isoforms, resulting from allelic polymorphism, are generated. One is truncated and non-functional, and the other is complete and expressed on NK cell surface [282, 283].

3.3.4.8. Other Activating Receptors

i. CD7

CD7, belonging to the Ig superfamily, is expressed on the surface of all NK cells and T cells. Its specific ligand, K12, is a protein secreted by epithelial cells [284, 285]. In rADCC experiments using an anti-CD7 antibody, an enhancement of NK cell cytotoxicity, IFN-γ secretion, and proliferation was observed [286, 287]. CD7 classification as a co-activation receptor is based on the finding that NK cells in CD7-KO mice are normal in quantity and cytotoxicity function [288]. Milush et *al.* identified novel subsets of peripheral blood (PB) CD3/14/19^{neg} NK cells and monocyte/DC-like cells. In fact, using CD7 and CD56 as surface markers, it is possible to distinguish two different cell types: CD7^{neg}CD56^{+} monocyte/DC-like cells, lacking the expression of NK cell-associated markers as well as NK cell functions, and CD7^{+}CD56^{+}, common NK cells [289].

ii. CD27

CD27 is a co-stimulatory molecule, a type I transmembrane glycoprotein, belonging to the TNF receptor superfamily [290] which recognises another member of the same family, CD70 [291, 292]. In mice, CD27 interaction with its ligand, or the use of an anti-CD27 antibody (ADCC) increases NK cell secretion of IFN-γ, but not their cytotoxicity [292]. Combined to CD11b, CD27 defines different stages of murine NK cell differentiation (*cf. chapter 3.4.2. Late Stages of NK Cell differentiation*) [293, 294]. In humans, more specifically in T cells, CD27 is a co-stimulatory receptor that enhances signals through the T cell receptor (TCR) upon engagement with its ligand, CD70 [295]. Loss of CD27 on human and murine T cells was correlated with an increase in their cytotoxic capacity [296]. In human, by using CD27, NK cells can be sub-divided into two main subpopulations differing in their compartmentalisation. This subdivision overlaps with the expression of

CD56: high or positive expression of CD27 on $CD56^{bright}$, and low to negative expression of CD27 on $CD56^{dim}$ NK cells [297, 298].

iii. CD69

CD69 is a type C lectin expressed as a disulfide-linked homodimer on most of the hematopoietic cells. Absent from non-activated NK cells, CD69 is a functional triggering molecule on activated NK cells [299]. In rADCC tests, an anti-CD69 induces NK cell cytotoxicity, proliferation, and cytokine production [300].

iv. DNAM-1 or CD226

DNAM-1 is a surface glycoprotein belonging to the Ig-superfamily and expressed on human NK cells, platelets, monocytes and a subset of T cells. It binds to CD155, a poliovirus receptor, and CD112, a nectin adhesion molecule [301] which are both upregulated on tumour cells. A physical and functional association between DNAM-1 and both, the adhesion molecule Lymphocyte function-associated antigen 1 (LFA-1), and the tyrosine-kinase Fyn was found [302]. This association is necessary for DNAM-1 mediated NK cell triggering. DNAM-1 plays an important role in spontaneous formation prevention and growth controlling of tumour cells [248, 303, 304]. NK cells also use DNAM-1 in their cross-talk with DCs since the latter cells express this receptor's ligands [305].

v. CD244 or 2B4

CD244 (2B4), expressed in human and mice, is a member of the Signalling Lymphocyte Activation Molecule (SLAM) receptor family [306]. Contrary to other SLAM receptors, 2B4 does not bind to itself in *trans*, it recognises another Ig-like hematopoietic molecule, CD48. In their cytosolic tail SLAM receptors present a tyrosine based motif (S/TxYxxL/I), similar to the ITIM motif, and called Immunoreceptor Tyrosine-based Switch Motif (ITSM) [307]. ITSMs transduce an activation signals through the SLAM-associated proteins (SAP) followed by the recruitment of the tyrosine kinase Fyn. 2B4-dependant activation signal in NK cells is ensured by two different ways: VAV-1 phosphorylation by Fyn, and SH2 recruitment blocking [308, 309]. Another SAP adaptor molecule is expressed in NK cells, Erwing's sarcoma-associated transcript-2 (EAT-2), which does not bind Fyn, but combines to SAP in NK cell activation upon 2B4 recognition of CD48 [310]. An inhibitory function is also attributed to the 2B4 receptor. This inhibition occurs during lymphocytic choriomeningitis virus (LCMV) infection in mice and protects activated $CD8^+$ T cell against NK cell-mediated killing [311]. The balance between both activation and inhibitory

functions of 2B4 receptor are still unknown.

vi. NKp80

NKp80 belongs to the C-type lectin family and presents in its cytosolic part two ITIM-like motifs [312]. It is expressed as a homodimer on all NK cells (activated or not) as well as $CD56^+$ T cells [313]. NKp80 binds to the genetically linked activation-induced C-type lectin (AICL) and stimulates NK cytotoxicity by using an atypical hemi-ITAM and the Syk-kinase pathway [314]. This interaction promotes cross-talk with monocytes, and increases the responses of effector memory $CD8^+$ T cells (4, 5).

3.3.4.9. Activating Receptors and their Synergistic Combinations

To secrete cytokines or kill target cells, NK cells require activation signals transmitted through their AR. But is each of the above mentioned receptors sufficient for triggering activation?

rADCC experiments, with antibodies against each of these receptors, showed that only CD16 cross-linking can lead to degranulation in resting NK cells [246] whereas other receptors (NCRs, NKG2D, DNAM-1 and 2B4) were found to activate NK cells only in combination with others. The rADCC resulting activation is different from the receptor/ligand interaction occurring *in vivo*. One solution would be to express the corresponding ligand on *Drosophila* cells, or attaching it to an artificial lipid bilayer. In such experiments, results similar to rADCC were obtained: not one single natural cytotoxicity receptor was able to induce degranulation alone. Specific AR synergistic combinations were important for such activation [315]. These experiments show that the NCRs, NKG2D, DNAM-1 and 2B4 have equal activation status, at least in degranulation (Fig. 13).

AR ligation and its effect on cytokine secretion was found to be more complicated and different than degranulation. Some receptors ligation were enough to induce IFN-γ secretion, like 2B4. This effect was additive, meaning that the ligation of more than one receptor could increase the cytokine secretion [155].

Receptor *(ligand)*	Degranulation		Polarisation	Killing
NKG2D *(ULBP1)*	No		No	No
2B4 *(CD48)*	No		No	No
NKG2D + 2B4	Yes[a]		No	No
LFA-1 *(ICAM1)*	No		Yes[b]	No
NKG2D + 2B4 + LFA-1	Yes		Yes	Yes
CD16 *(anti-S2 IgG)*	Yes		No	No
CD16 + LFA-1	Yes		Yes	Yes
NKG2D + 2B4 + IR[c] *(HLA-E)*	No[a]		No	No
LFA-1 + IR *(HLA-C, HLA-E)*	No		No	No
CD16 + IR *(HLA-E)*	Less		No	No
CD16 + LFA-1 + IR *(HLA-C, HLA-E)*	Yes	Less[d]	No	No

[a]Green shading indicates activation, red shading indicates inhibition.
[b]In mouse NK cells, polarisation requires co-engagement of LFA-1 with NKG2D
[c]Inhibitory receptors (KIR and/or CD94/NKG2A)
[d]Tested with NK clones; some were partially inhibited

Figure 13: NK cell activating receptors (AR) synergistic combinations.

Shown is a table summarising NK cell responses following their activating receptors (AR) engagement with their specific ligands expressed on insect S2 cells. Not one single AR was able to induce a complete NK cell response, demonstrating the AR synergistic combination necessity for NK cell degranulation and target cell killing. Adapted from [6], no permission needed.

3.3.5. Adhesion Molecules (AM)

NK cells express a large number of AM like, CD2, CD11a/CD18 (LFA-1), CD11b, CD31, CD44, CD49d/CD29 and CD62L. These molecules are implicated in different functions: endothelial adhesion, extravasation, migration, homing, tissue infiltration, conjugates formation with target cells, and cytotoxic activity co-stimulation [316]. In this part, we will develop the role of some of the above mentioned AM.

i. CD2

CD2 is a surface protein belonging to the Ig superfamily, expressed by human T cells and a subset of NK cells. It binds CD48, and CD58, a ligand widely expressed on hematopoietic cells [317, 318]. Only a fraction of $CD2^+$ NK cells that does the engagement of CD2 molecules with their ligands can result in NK cell activation. Such observations suggest that CD2 is a co-receptor during NK cell mediated cytotoxicity [319-321]. This was

explained by the fact that CD2 plays an important role in nanotubes' formation on NK cells, enabling them the exploration of their local environment and interaction upon target cell encounter [322].

ii. LFA-1 or CD11a/CD18

LFA-1, or leukocyte function associated molecule-1, is a heterodimer, expressed by all leukocytes, and formed of the two integrins, α_L and β_2 (CD11a and CD18 respectively) [323]. LFA-1, or CD11a/CD18, binds three different AM: intercellular adhesion molecule (ICAM)-1 (CD54), ICAM-2 (CD102), and ICAM-3 (CD50) [324]. LFA-1 play an important role in the NK cell cytotoxicity mechanism [316]. NK cells derived from LFA-1 KO mice are phenotypically normal, present normal cytokine secretion properties, and have the necessary CG for degranulation, but upon target cell encounter less conjugates are formed (60% to 80% reduction), and their cytotoxicity activity is reduced [325]. This cytotoxicity requires both cells (NK and target cell) adhesion, which is mainly mediated by the interaction of LFA-1 and its ligand, ICAM-1 [326]. Such a cytotoxicity reduction was also observed when LFA-1 was blocked by an antibody [327]. LFA-1 was found to promote degranulation but not polarisation [146, 315].

iii. CD11b (MAC-1)

Like LFA-1, CD11b (or macrophage-1antigen (MAC-1)) is part of the heterodimeric integrin $\alpha_M\beta_2$ molecule, β_2 being CD18. In mice, CD11b devises NK cells into two subsets, $CD11b^{low}$ subset, present in foetal and neonatal mice [328] as well as adult mice's BM, and $CD11b^{high}$ NK cells, present in peripheral sites, such as spleen, peripheral blood mononuclear cells (PBMCs), and lung [329]. Along with CD27, CD11b determines four different stages of murine NK cell maturation [294], (*cf. chapter 3.4.2. Late Stages of Differentiation*). In human, nearly all PBMC NK cells are $CD11b^{high}$. By comparing these NK cells (phenotypically and functionally) to the decidua NK cells, known to be less mature and having a $CD11b^{neg}$ $CD27^{neg}$ phenotype, it was proposed that both markers, CD11b and CD27, can determine, like in mice, different human NK cell differentiation steps [330].

iv. CD56

CD56, a binding glycoprotein, is an isoform of the neural cell adhesion molecule (NCAM), expressed in human but not in mice. This AM is expressed on the surface of neurons, skeletal muscle and NK cells [331-333]. Although CD56 is the main human NK cell marker, it does not seem to be implicated in these cell's functions and its role is still unknown. The

use of anti-CD56 antibodies does not induce NK cell activation, nor inhibition, on the contrary, it is used as a negative control when studying other receptors' effect upon antibody recognition [323, 334]. NK cell subpopulations' division is based mainly on the CD56 expression into two main subsets: $CD56^{bright}$ and $CD56^{dim}$. In response to interleukin stimulation, $CD56^{bright}$ cells produce IFN-γ and proliferate, whereas $CD56^{dim}$ produce cytokines and become cytotoxic upon their AR triggering [335-337]. After few days of IL-2 activation, CD56 density increases on $CD56^{dim}$ NK cells. A third population, $CD56^{neg}$ $CD16^{+}$, rare in healthy donors, represent up to 40% of NK cells in HIV-1 and hepatitis C virus (HCV) patients [338, 339]. Phenotypically, $CD56^{neg}$ cells express NKp46, at low levels under certain conditions, as well as molecules which are associated with NK cells [340]. This third subset shares the phenotype features of immature NK cells, high expression of CD94/NKG2A and low expression of CD57, but also of mature NK cells, high expression of KIR molecules [341, 342]. Functionally, they are skewed towards cytokine production suggesting a role in virus infection control [340]. All these characteristics make $CD56^{neg}$ $CD16^{+}$ cells a possible third subdivision of NK cells, thus reducing the specificity of CD56 and calls for the use of multiple approaches to reach the NK cell definition.

v. CD62L

CD62L, a member of the selectin family (L-selectin), is a homing molecule important for the recruitment of NK cells to the lymph nodes [343]. It can also initiate leukocyte tethering while rolling along the endothelium [344]. CD62L expression was suggested to be a part of the NK cell development in mice and humans. Kerstin et *al.* have shown that human NK cell development can be divided into three different steps using CD56 and CD62L: $CD56^{bright}$, $CD56^{dim}$ $CD62L^{+}$ and $CD56^{dim}$ $CD62L^{-}$, where the $CD56^{dim}$ $CD62L^{+}$ is the only subset retaining both $CD56^{bright}$ and $CD56^{dim}$ functions (cytokine production and cytotoxicity) [345]. In mice, CD62L is expressed at higher levels in $CD11b^{+}$ than $CD11b^{neg}$ NK cells, suggesting a correlation between its expression and NK cell development [293].

3.3.6. Other NK Cells Phenotypic Markers

i. CD8

CD8 is a transmembrane glycoprotein belonging to the Ig superfamily. Two different isoforms were identified, CD8α and CD8β, encoded by two different genes. While CD8α is associated with a short cytosolic tail that interacts with a SRC-like tyrosine kinase,

p56lck, CD8β's 15 aa cytosolic tail has no identifiable protein binding motif [346, 347]. CD8 is expressed on the surface of T cells, in both human and mice, mainly as a disulfide-linked heterodimer composed of a CD8α and CD8β chain, and serves as a co-receptor for the T cell receptor (TCR) when binding to MHC class I molecules [348-350]. While it is absent on resting murine NK cells, CD8 is expressed on a subset of human NK cells, and a small subset of T cells, as a CD8α/α homodimer [351, 352]. The interactions of both CD8α/α and CD8αβ with the MHC class I molecules were found to have identical affinities [347, 353]. Functionally, resting and IL-2-activated human $CD8^+$ NK cell subsets were found to be more cytotoxic than their $CD8^-$ counterparts [354, 355]. Addison *et al.* have shown that $CD8^+$ NK cells are less prone than $CD8^-$ NK cells for apoptosis after target cell ligation and lysis, allowing them to perform multiple target cell killing [356].

ii. CD57

CD57 represents a carbohydrate epitope containing a sulfoglucuronyl residue. The enzyme processing this epitope is beta-1,3-glucuronyltransferase 1 (B3GAT1), a type 2 transmembrane protein with a relatively short cytoplasmic region [357]. This epitope is widely expressed in mouse brain [358], on human neurons and retina cells [359] as well as on NK cells, subsets of T cells, B cells and monocytes. In T cells, CD57 is a marker of high differentiation, indicating cells capable of IFN-γ and IL-5 production and cytotoxicity, but reduced proliferation [360]. Only $CD56^{dim}$ NK cells express CD57, an expression which increases by age [361]. In fact, $CD57^+$ $CD56^{dim}$ NK cells, compared to their $CD57^-$ counterparts, express less CD94/NKG2A, CD27, CD62L, NKp30, NKp46 and NKG2D, and more KIR molecules. Functionally, $CD57^+$ NK cells produce less IFN-γ than $CD57^-$ NK cells, the opposite being observed in ADCC assays. As for the proliferation capacity and response to IL-2 and IL-15, the expression of CD57 makes NK cells less prone to these functions. All these characteristics, which occur independently from NK cell education, associate the expression of CD57 on NK cells with a unidirectional differentiation, leading to a final maturation step [361, 362].

vii. CD94

CD94 is a glycoprotein, part of the C-type lectin superfamily, and has a very short cytosolic tail that does not transduce any signal. It can be disulfide-linked with the different inhibitory and activating members of the NKG2 family (NKG2A, B, C, E, and H), but not with NKG2D [198]. The CD94/NKG2 complex recognises HLA-E in human and Qa-1 molecule in mice. CD94 is considered to be an invariant chain serving as a chaperone for the NKG2

receptors' transport to the cell membrane [363] and is acquired during the late stages of NK cell development. The depletion of KLRD1 gene in mice (the CD94 gene), did not affect NK cell development and maturation, nor their cytokine production and lytic functions against the YAC-1 target cell line [364]. Like CD62L [345], CD94 was reported to subdivide human NK cell into 3 distinct subsets according to their maturation process: $CD94^{high}$ $CD56^{bright}$, more potent in IFN-γ secretion, less cytotoxic, and expressing less perforin and Grz B than their $CD94^{low}$ $CD56^{dim}$ counterparts, and $CD94^{high}$ $CD56^{dim}$, showing intermediate properties between the two latter cell types [365]. This subdivision was also proposed by Moretta *et al.* where CD56, CD94, KIR, CD16, CD57 and perforin were used to identify four different stages in NK cell transition from $CD56^{bright}$ to $CD56^{dim}$ [366].

3.4. NK Cell Development and Differentiation

In mammals, PBMC derive from bone marrow (BM) self-renewing pluripotent hematopoietic stem cells (HSCs) which give rise to the common myeloid progenitor (CMP) and the common lymphoid progenitor (CLP). A series of maturation and differentiation steps, following various hierarchical fashion pathways, leads to functionally competent cells. Thrombocytes, erythrocytes, mast cells, basophils, neutrophils, eosinophils and monocytes are derived from the CMP, whereas T cells, B cells, and NK cells are derived from the CLP. Dendritic cells can be derived from both progenitors, CMP and CLP [367].

NK cell development studies constitute a large area of investigation. The elucidation of the immunophenotypes of the different NK cell developmental intermediates (NKDIs) is of high importance as NK cell application in cancer therapy, and other diseases' treatment, is growing by time. NK cell development, which starts by the HSCs differentiation, is divided into 6 different stages, based on multi-parameters' cell surface markers [368] (Fig. 14A, B). In this section, we will present the main cellular intermediates, the developmental pathways as well as the anatomical sites of human and mouse NK cell development.

3.4.1. Early Stages of Differentiation

NK cells, like all lymphocytes, are derived from BM HSCs. In humans, HSCs and CLP are part of the $CD34^{+}$ BM population and defined as lineage negative [367]. During the early stages of development, stages 1, 2 and 3 in humans, and, CLP, pre-NK precursors (pre-NKP) and NK precursors (NKP) in mice, NK cells become gradually committed to the NK cell lineage. An important step in both human and murine NK cell development is the acquisition of the

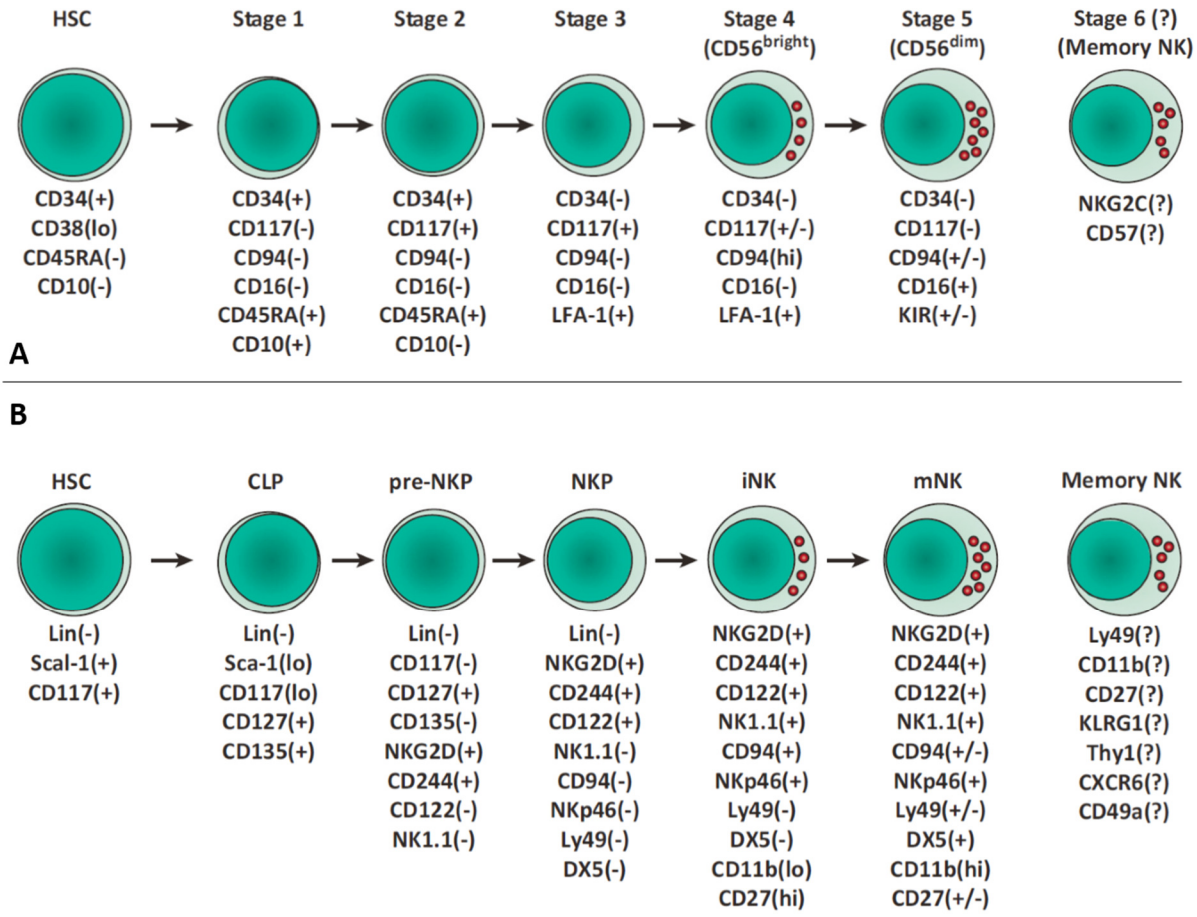

Figure 14: Representation of the different NK cell developmental intermediates (NKDI) in human and mice.

(**A**) Human NK cell development from hematopoietic stem cells (HSCs) to mature CD56^{dim} ones (stage 5) is represented as a linear pathway. Memory NK cells (stage 6) are depicted here as one possible terminal stage of NK cell development/maturation, although different pathways are known to lead to this stage. (**B**) Mouse NK cell development from hematopoietic stem cells (HSCs) to mature CD11b^{high}DX5^{+} NK cells is represented as a linear pathway. As in humans, memory NK cells are depicted here as one possible terminal stage of NK cell development/maturation, although different pathways are known to lead to this stage. Shown under each developmental stage, different surface markers which can be used to distinguish NKDI population. Abbreviations: CLP, common lymphoid progenitor; iNK, immature NK cell; mNK, mature NK cell; NKP, NK cell precursor. Expression is designated as '+' (expression), '–' (no expression), 'hi' (high expression), or 'lo' (low expression). Adapted from [8], permission number 3326560423995.

interleukin (IL)-15 receptor (IL-15R) [369] more specifically the β chain, common with the IL-2R (cf. *chapter 3.4.3. i. Cytokines and Chemokines*).

The definition of early NK cell precursors is based on different characteristics. These cells lack the phenotypic features of mature NK cells, are unable to produce IFN-γ nor mediate cytotoxicity, and have, under IL-15 stimulation, the potential to develop into NK cells [370, 371].

Committed NKDIs are cells lacking lineage specific antigens (like CD3, CD14 and CD19) with the potential of becoming NK cells but no other hematopoietic cell lineages (T cells or DCs). These cells were believed to belong to the stage 3 of differentiation as the latter stage cells are not able to revert to the previous stage 2 NKDIs [370]. Recent studies showed an overlapping between the stage 3 committed NKDIs and some ILC populations in the human secondary lymphoid tissue (SLT) [372]. The main difference between these two cell types are the specific development requirements. ILCs require the nuclear receptor RORγt for their development, whereas NK cells never express this gene during their *in vivo* lifespan [373]. Another factor, IL-7, is essential for ILC3 development, but not NKDIs, the opposite being true for IL-15 [374].

3.4.2. Late Stages of Differentiation

During the next 3 stages of NK cell development, stage 4 ($CD56^{bright}$), 5 ($CD56^{dim}$) and 6 (memory NK cells) for human, and immature NK (iNK), mature NK (mNK) then memory NK for mice, NK cells undergo functional maturation, become cytotoxic, and gain the ability to secrete different cytokines and chemokines. The acquisition of the different receptors, in both humans and mice, follows a certain specific order. In humans, CD161 is acquired first, followed by CD56, CD94/NKG2A, NKp46 then KIR and CD16 [119, 171, 361, 375]. Mouse NK cells follow a parallel expression order: NK1.1 is the first to be expressed, CD94/NKG2A, NKp46 then the Ly49 receptor family and finally CD11b [329]. During their lifespan, NK cells partially downregulate or lose some of these receptors, like CD56 in humans, CD27 in mice, and CD94/NKG2A in both species [376]. Based on their functionality, mature NK cells, in both species, can be divided into at least two different populations.

Human $CD56^{bright}$ and $CD56^{dim}$ are the main two NK cell populations [336, 377]. While the $CD56^{bright}$ subset is more potent for cytokine production upon soluble factors' stimulation, $CD56^{dim}$ cells represent the main cytotoxic NK cell population, and are capable of strong cytokine response resulting from a cell surface ligands engagement [6, 119]. NK cell differentiation, as well as telomere length studies indicate that $CD56^{bright}$ are the precursors of $CD56^{dim}$ NK cells [378-380]. This notion is supported by the fact that the $CD56^{bright}$ subset is the first to appear in PB following stem cell transplantation [381] whereas $CD56^{dim}$ NK cells appear later and accumulate during life [362]. Other receptors were also investigated and found to have a certain progression pattern during NK cell maturation, like CD94, CD62L, CD27, NKG2A, KIR and CD57 [345, 361, 362, 365].

Among the mNK in mice, there can be determined different subpopulations with different functional competence. Stimulation of $CD27^{high}$ murine NK cells leads to a higher cytokine production and cellular cytotoxicity compared to their $CD27^{low}$ counterparts [382]. The notion that, like human $CD56^{bright}$ and $CD56^{dim}$ NK cells, $CD27^{high}$ NK cells are $CD27^{low}$ precursors, was

confirmed by adoptive transfer experiments, showing that $CD27^{high}$ can give rise to $CD27^{low}$ NK cells, the opposite not being possible [382]. These data lead to a 4-steps murine NK cell maturation pattern based on CD11b and CD27 expression: $CD11b^{low}/CD27^{low}$ → $CD11b^{high}/CD27^{low}$ → $CD11b^{high}/CD27^{high}$ → $CD11b^{low}/CD27^{high}$ [294].

Traditionally, immunological memory is restricted to T cells and B cells, NK cells being considered as part of the innate immunity with short lifespan. In the past few years, several studies provided evidence of specific NK cell features of adaptive immune memory induced by MCMV infection [383, 384], exposure to cytokines [385], and a liver-restricted NK memory cells directed towards specific antigens [386].

3.4.3. Factors Influencing NK Cell Differentiation

During their development and differentiation process, NKDIs are subject to many factors which play an important role. These factors can be extrinsic, like cytokines, chemokines and the surrounding environment, or intrinsic, like transcription factors.

i. Cytokines and Chemokines

By directing the NKDIs into specific tissues and distinct microenvironments, chemokines play an important role in NK cells differentiation and maturation [387]. Most of the available data dealing with the role of cytokines and chemokines on NK cell development were the result of *in vitro* studies. It is still unclear when, how and at what concentration these molecules play their role *in vivo*.

One of the most important IL in NK cell development is IL-15, and its action through its receptor IL-15R, downstream the CLP differentiation [369, 388]. This receptor is composed of 3 subunits: the high affinity α chain (CD215), the β chain (CD122) which is shared with IL-2 (IL-2/IL-15R), and the common γ chain (CD132) present in IL-2, -4, -9, -15 and -21 receptors [389]. While *in vitro* the soluble IL-15 drives NK cells development from BM-derived HPCs [390], *in vivo* IL-15 is coupled to CD215 then presented *in trans* to the CD122 and CD132 expressing cells [391].

Some cytokines, like flt3 ligand can play an indirect role in NK differentiation by promoting expansion of an important IL-15 source, DCs [392]. The tyrosine kinase receptor Axl/Gas6 (adhesion-related kinase/growth arrest-specific gene 6) positively regulates flt3 and thus participates in NK cell development [393]. In contrast, other cytokines are known to repress or decrease the development of NK cells, like TGF-β [394]. IL-1β, in the presence of IL-15 favours the expansion of ILC3 compared to NK cells [395].

ii. Environmental Cells

NK cells can be found in most tissues, as well as blood. In human and mice, these tissue resident NK cells are shaped by their environment and may present unique roles during the immune response as well as tissue integrity. In case of malignant cell growth, infection or inflammation, specific PB NK subsets can be recruited to the concerned organ and perform their specific and necessary functions. For a long time it was thought that CLP were only present in BM, but $CD34^+$ precursors were found in many other tissues, like thymus [396], cord blood (CB), SLT, lymph nodes [397] and blood. These cells can, *in vitro* and under IL-2 or IL-15 stimulation, differentiate into mature NK cells [171, 398, 399], an interaction mediated by the IL-2/IL-15R β chain, and the common γ chain. BM stromal cells were reported to play an important role in NK cell differentiation, mediated by receptor-ligand interactions between these two cells. Deletion of the three Tyro3 family receptor members (Axl, Tyro3 and Mer), expressed either on NK cells or stromal cells, resulted in a NK development and function alteration [400]. T cells, DCs and monocytes were also found to mediate NK cell differentiation by secreting IL-15 and/or IL-2 [401, 402]

iii. Transcription Factors

Transcription factors (TFs) are important players in the hematopoietic lineage specification [403, 404]. E4BP4/NFIL3, a basic leucine zipper transcription factor, plays a role in different immune processes [405]. In E4BP4-deficient mice, NK cells number are highly reduced in different organs (LN, liver, spleen and BM) indicating that E4BP4 is absolutely required for NK cell development in the BM [406, 407]. Ets-1, known to play a role in T cell and B cell development, is critical for NK cells differentiation as it is implicated in the very early stages of development by inducing other TFs expression [408]. Ets-1 KO mice showed a normal CLP count, but NK cell committed precursors number was decreased leading to a 5-fold to 10-fold reduction in mature NK cells' number [408]. Helios, belonging to the Ikaros zinc-finger family of TFs, is highly expressed in murine $CD11b^{neg}$ NK cells. Studies on $Ncr1^{Noé/Noé}$ mice suggested a possible link between the up regulation of Helios and the absence of NKp46 expression [247]. Splenic NK cell maturation, but not specification, was found to be stopped in GATA-3 KO mice. While immature GATA-3 KO splenic NK cells had normal cytotoxicity, the secretion of IFN-γ was reduced compared to WT NK cells [409]. GATA-3 was also reported to be indispensable for the generation of murine $CD127^+$ thymic NK cells [396]. Loss of KLF4 affected NK cell number in PB and spleen but not in the BM, Lymph Node (LN) and liver. Functionally, only splenic NK cells showed increased apoptosis. Interestingly, these defects were compensated after

adoptive transfer of KLF4-deficient NK cells in WT mice [410].

3.5. NK Cell Education

In contrast to T and B cells, the term “education”, for NK cells, does not systematically imply a defined development mechanism taking place in the thymus or BM, but more the adaptive process(es) that a NK cell undergoes to achieve functional competence. This process(es) results from the NK cell interaction with MHC class I molecules, and allows it to sense the absence or differences in these molecules’ expression on surrounding cells, termed “missing-self”. To achieve such functions, the IR expression on NK cells should be regulated in a way such that NK cells can recognise and eliminate the non-self or altered-self, while remaining tolerant to the autologous (self) cells.

NK cell educational mechanisms have not been yet entirely elucidated, and are still intensively debated. In this section we will discuss the different models of NK cell education, including the original one, the missing-self hypothesis and how it gave rise to the other models, as well as the role of MHC class I molecules (Fig. 15).

3.5.1. Missing-self hypothesis

Early studies by Georges Snell in 1958 [411, 412], demonstrated that following a bone marrow transplantation, an irradiated F1 hybrid individual (with a genotype A/B) rejects the tissue if derived from AA or BB genotype individuals, a rejection controlled by MHC class I molecules. Years later, Cudkowicz *et al.* [413] showed that such tissues’ rejections are controlled by a bone marrow derived and thymus independent cell type. Such observations correlate with the initial NK cell description and led K. Kärre to elaborate the missing-self hypothesis in 1981: the absence or reduced expression of MHC class I molecules would render target cells susceptible to NK cell attack. Mechanistic models explaining the initial hypothesis were published in the following years by K. Kärre’s team, suggesting the existence of unknown structures allowing NK cells to detect; (i) the self and transmitting negative signals, or (ii) the absence of self (missing-self) resulting in a triggering of the NK cell which could lead to the target cell lysis [118, 414]. The work of L. and A. Moretta, starting in 1994, allowed the identification of different NK cell IR and AR as well as their ligands, HLA class I molecules [415-420]. In 1997, Valiante *et al.* [421] showed that a NK cell should express at least one self-specific MHC class I receptor to be able to detect the presence or absence (missing) of self.

During the following years, the identification of NK cell subsets lacking the expression of IR or any MHC class I receptor [422, 423], as well as the different requirements for a correct NK

cell function, took the NK cell education beyond the missing-self hypothesis. Although the missing-self hypothesis has been proven to be incomplete, it provided an important platform for the generation of other models.

3.5.2. Licensing, or Arming Model

In a mouse model, on the C57BL/6 background, the comparison of Ly49C$^+$ NK cells (which bind H2-K^b, a self-MHC class I ligand), showed that they are more cytotoxic and produce more cytokines than NK cells expressing only Ly49A (which has no MHC class I ligand in the C57BL/6 mice) [424]. The same results were observed on human NK cells expressing KIR3DL1 and its ligand, HLA-Bw4 [425]. An explanation for such differences is that IR provide an ITIM-dependent instructive signal to the NK cells rendering them responsive, or "licenced", "armed" [424, 426]. NK cells are initially hyporesponsive and their AR are inactive. When a NK cell acquires a self-specific IR, the signal transmitted by its ITIM motif instructs them and renders their AR competent, giving NK cells their responsiveness. Hyporesponsiveness caused by a lack of ITIM-dependent signalling can be overcome by cytokines [424]. During inflammation, the NK cell licensing lack is not a handicap, as the secreted IL can induce NK cell activation and overcome their hyporesponsiveness, such as the case with MCMV infection [427, 428].

The licensing model is compatible with the hybrid resistance, described by Georges Snell [411, 412]. In a F1 hybrid individual, H2-K^{b}D^d, Ly49A$^+$ and Ly49C$^+$ NK cells are separately licensed by both MHC class I molecules, H2-K^b and H2-D^d respectively. Once they encounter a parental cell, H2-K^b or H2-D^d, the lack of one of the two MHC class I molecules will be detected, and the NK cell will attack the corresponding cell, causing the rejection. The work of Elliott *et al.* [429] in 2010 provided another proof of the licensing model. When transferred into a MHC class I sufficient environment (WT mice), MHC class I deficient (β_2m KO) NK cells lost their hyporesponsiveness and exhibited functional properties comparable to NK cells derived from WT mice. Such results suggest that neither exposure to MHC class I ligands during NK development in the BM nor endogenous MHC class I expression by NK cells themselves is absolutely required for licensing (*cf. chapter 3.5.5. Cis and Trans Interactions, and chapter 3.7. Trogocytosis*)

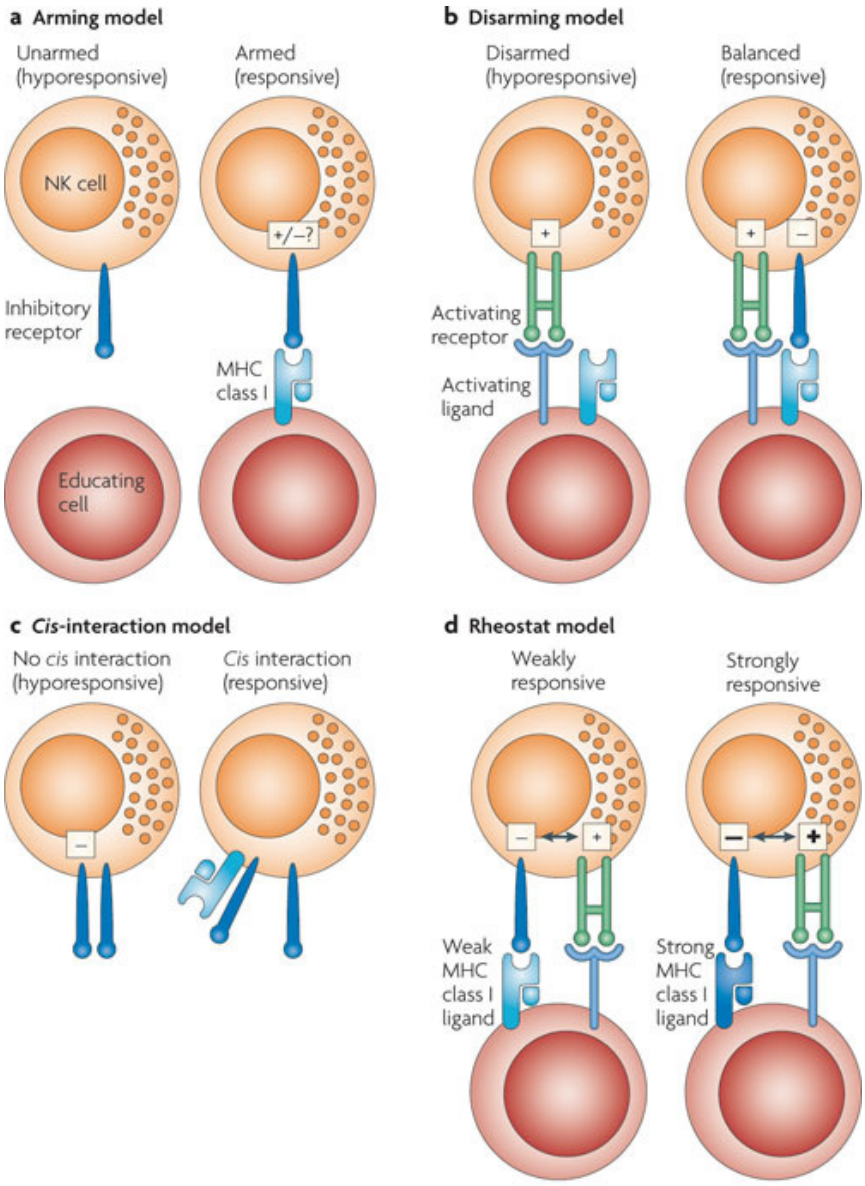

Figure 15: NK cell education models.

Depicted are the different NK cell education models. In the arming model (**a**), NK cell functional competence is ensured by the inhibitory receptor (IR) signalling: NK cells are hyporesponsive in the absence of this inhibitory signal. (**b**) In contrast, according to the disarming model, NK cells are initially activated. In the absence of a self-MHC class I specific IR, the NK cell becomes hyporesponsive. In the *cis* interaction model (**c**), NK cell education is ensured by the interaction of IR with its cognate MHC class I molecule in *cis*, that is on the same cell membrane. Such binding can reduce the available IR that can interact in *trans* in the immune synapse, thus making NK cells responsive. The rheostat model (**d**) is based on the inhibitory signal strength during NK cell education. The activating threshold is balanced as a rheostat, allowing the NK cell maturation to be optimally tuned by the inhibitory input, making the rheostat model a dynamic NK cell education mechanism. Taken from [9], permission number 3356030822296.

3.5.3. Disarming Model

Contrary to the licensing model, the disarming model highlights the effect of activation on NK cell education, in other words, NK cell responsiveness is tuned down. Hyporesponsiveness is an induced state, caused by an overstimulation of NK cells that present a lack in inhibitory signalling [422]. Initially, NK cells are responsive and have their functional competence retained by the activation pathways, allowing them to respond in the case of missing- or altered-self. When NK cells fail to ensure the expression of a self-specific IR, the continuous stimulation results in a withdrawal of their responsiveness, leading to a state of hyporesponsiveness [430]. In this model, the only requirement of IR is to do their assigned job; inhibiting NK cells in the presence of their ligands, MHC class I molecules.

Consistent with this model, the AR NKG2D became dysfunctional after an *in vitro* prolonged exposure of NK cells to tumour cells expressing a ligand for NKG2D [431]. This chronic engagement of NKG2D did not only affect the latter receptor, but cross-tolerised other receptors with activating pathways independent of NKG2D, like NK1.1 and CD16 [431]. Similar observations were made when Ly49H^{+} NK cells were transferred into a transgenic mouse model expressing the MCMV-encoded peptide, m157. Responsiveness of Ly49H^{+} NK cells was found to be reduced although they express IR and MHC class I molecules at normal levels [432, 433]. The adoptive transfer of mature responsive NK cells into MHC class I deficient mice (β_2m KO mice) resulted in their loss of responsiveness, providing another evidence in favour of the disarming model [107]. Such results presume that NK cells, once chronically exposed to MHC class I-deficient host cells, may develop a state of induced hypo-activity. NK cells derived from a D^{d} transgenic H-2^{b} mouse model, in which some cells do not express the D^{d} transgene, were unable to kill D^{d-} target cells [434, 435]. In contrast, murine NK cells chronically exposed to tumour cells, or MHC class I deficient splenocytes, were unable to become hyporesponsive *in vivo* [431].

In the above mentioned situations, by adding cytokines and culturing them *in vitro* in the absence of the stimulatory cells, hyporesponsive NK cells gained their functions [431, 432, 434, 435]. This clearly shows that hyporesponsiveness is reversible and needs continuous stimulation in order to be maintained.

3.5.4. Rheostat Model

The main differences in the two NK cell education models (licensing and disarming) is the default/initial state of uneducated NK cells. NK cells can have their functions (i) gained and licensed by inhibitory signals, or (ii) withdrawn and disarmed following their over-stimulation in the absence of inhibitory signals. But these two models fail to answer one question, are all MHC class

I molecules equally efficient in the NK cell education process? Especially that both, human and mice, can express up to six different MHC class I molecules.

Using a murine transgenic system, the effect of H2-L^d, H2-D^d and their chimeric form, H2-D^d/L^d, on NK cell response was studied. The results showed that the chimeric form H2-D^d/L^d had the same impact as the H2-D^d transgene on NK cell response, although Ly49G can efficiently bind both MHC class I molecules [436]. These results were confirmed by a more detailed study investigating the effect of K^b, D^b, L^d and D^d as well as many chimeras on NK cell functions [437]. The four MHC class I molecules, when expressed individually, were able to educate efficiently NK cells. Once expressed as chimeras, the K^d and D^d molecules induced a better missing-self response than the D^b and L^d molecules. Not only MHC class I molecules have a strong effect on NK cell education, their receptor expression and ligation strength can also be determinant. Using a detailed multicolour flow cytometry staining, including functional readout, Brodin *et al.* showed that the IR co-expression influences the functional responses' results in an individual NK cell. In a mouse model expressing MHC class I molecules of low educating impact, IR co-expression lead to a synergistic effect on NK cell functions. The more a NK cell expresses self-specific IR, the better its missing-self response is [438, 439]. NK cell capacity of missing-self response is also directly correlated to the MHC class I expression level: once it drops to half, NK cell rejection of MHC class I-deficient cells also drops to half [438].

In the line of the above mentioned studies, both licensing and disarming models are validated, but fail to explain the quantitative feature of the NK cell education. A quantitative tuning model was proposed, the rheostat model, in which the NK cell can be tuned up (licensed) or tuned down (disarmed) during its development and adapt to its MHC class I environment [438]. MHC class I molecules can educate NK cells, and their interactions with their receptors (IR) sets the threshold for NK cell activation, resulting in a gradual responsiveness in which a higher inhibition leads to a higher and more efficient NK cell response. Such tuning can be perturbed during cellular stress, inflammation, or any other situation, leading to an efficient NK cell response.

3.5.5. *Cis* and *Trans* Interactions

The different models were able, in different ways, to give more insight to the tolerance/education of NK cells, but the molecular mechanism behind is still unclear. They all agree on the importance of the NK cell IR interaction with the surrounding cells' MHC class I molecules (in *trans*), ignoring another feature of these receptors: their interaction in *cis* with MHC class I molecules expressed on the surface of the same NK cell [219, 440-443]. The existence of these two binding capacities of the IR to the MHC class I molecules (Fig. 16A, B) lead to a questioning whether these two types of interaction have a role in NK cell education.

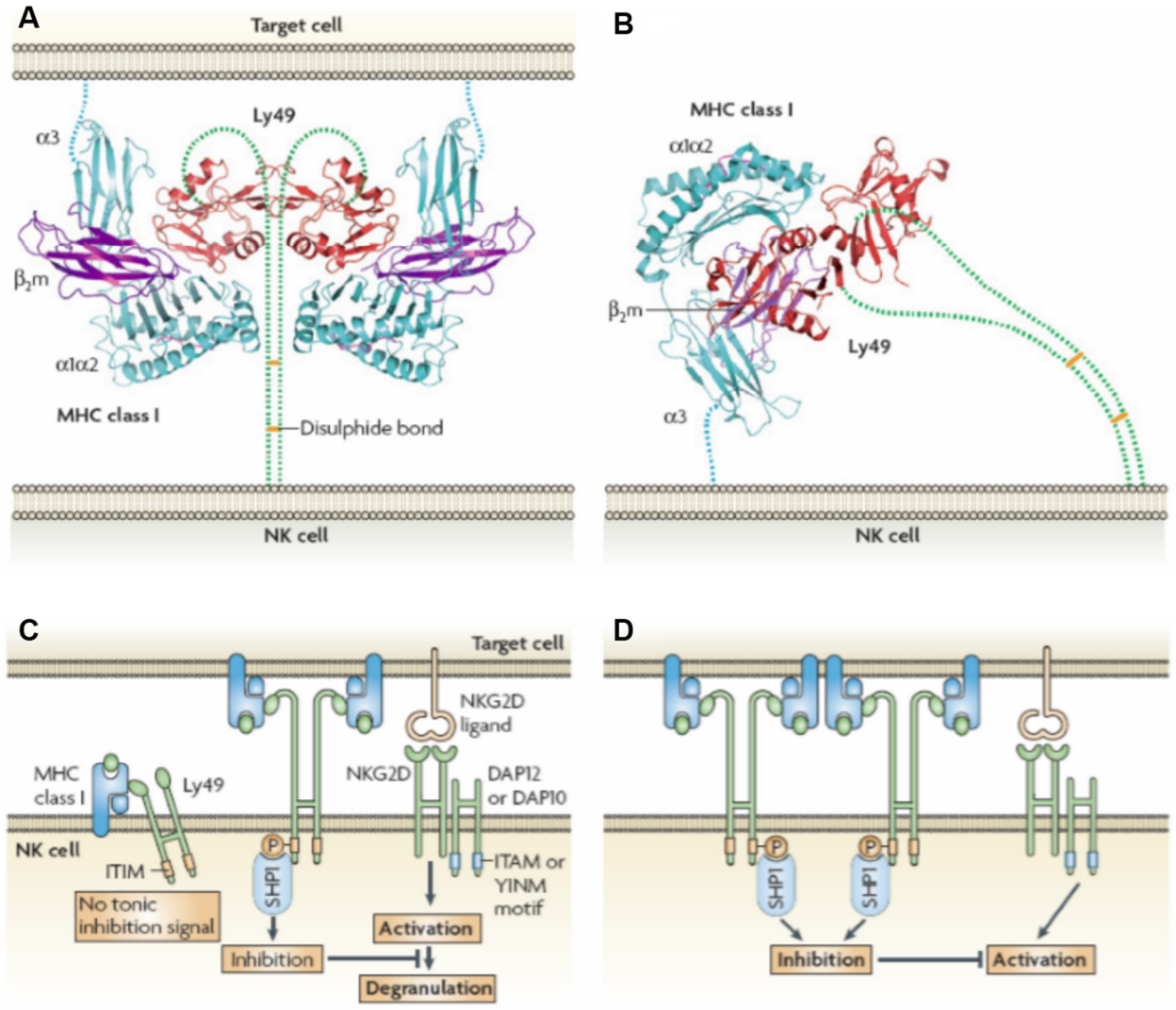

Figure 16: Cis and trans interactions between NK cell inhibitory receptors (IR) and their specific MHC class I ligands.

(**A** and **B**) Shown are the interactions of MHC class I molecules (H2-K^b) with Ly49C. Such interactions can occur in *trans* (**A**) when each of the two molecules is on different cells, or in *cis* (**B**) when both molecules are expressed on the same cell surface. By comparing both interaction types, stalk regions do not adopt the same conformation: back-fold conformation when in *trans*, and extended conformation when in *cis*. MHC class I molecule is shown in cyan, β2m in purple, Ly49C in red, and the stalk regions of both molecules (green) are represented in an arbitrary way as no crystal structure is available. (**C**) The association of IR with their specific MHC class I molecule in *cis* reduces the pool of available IR that can engage in *trans* with target cell MHC class I molecules. Following an encounter with a potential target cell, the balance between the inhibition and activation signals becomes more sensible, thus activating the NK cell and leading to the target cell lysis. When *cis* interactions are absent (**D**), trans interaction between NK cell IR and target cell MHC class I molecules leads to a strong inhibition signal. When a NK cell encounters a target cell, the activating signal may not be sufficient to overcome the inhibition signal, and may not induce target cell lysis. Adapted from [12], permission number 3326561281597.

To date, *cis* interactions have exclusively been demonstrated for MHC class I receptors with inhibitory functions, like the Ly49 family members on NK cells (the most studied one being Ly49A which binds H2-D^d) [219, 441-443] and the ILT receptor family on mast cells [444], whereas no evidence of such interaction between KIRs or CD94/NKG2 receptors and MHC class I molecules was found. It was shown that *cis* interaction, between Ly49A and H2-D^d, regulates the accessibility of the Ly49A receptor. (*cf. chapter 3.7. Trogocytosis*). This accessibility, measured by the anti-Ly49A antibodies, correlates more with NK cell licensing than *trans* interactions [219, 442]. In fact, the *cis* interaction modulates Ly49A accessibility thus regulating the NK cell inhibitory signal.

Once a NK cell encounters a target cell, the latter is killed when the activating signal exceeds the inhibitory one, meaning that the necessary threshold for NK cell activation is set by the inhibitory signal strength (Fig. 15C, D). In the case where the available IR number is limited, NK cell inhibition is consequently affected and can lead to target cell killing following a minor reduction of host MHC class I molecules' expression [445]. When NK cells, not expressing H2-D^d, interact with H2-D^{d+} target cells, Ly49A receptors are recruited to the immunological synapse where they achieve their role: inducing inhibitory signal following their engagement with their ligand [446, 447]. In contrast, when NK cells express H2-D^d, the Ly49A recruitment to the immunological synapse is reduced due to their interaction in *cis* with their ligand, leading to a higher NK cell responsiveness by restricting the inhibitory capacity of Ly49A [219, 440, 441].

By considering the *cis* interaction model, more insight can be given to some previous works' conclusions, notably the work by Elliott *et al.* in 2010 [429]. As exposed in the *chapter 3.5.2. Licensing, or Arming Model*, MHC class I deficient NK cells (β2m KO) were adoptively transferred into WT mice resulting in a gain of function for these NK cells. These results call for questioning the role of the endogenous expression of MHC class I molecules (which results in *cis* interactions) in NK cell education, but forget to take in consideration an important NK cell property, trogocytosis (*cf. chapter 3.7. Trogocytosis*).

3.6. NK cell's HLA class I Receptor Repertoire Formation and Evolution

It is clearly established that NK cell education is highly dependent on their IR expression, referred to "NK cell repertoire", and its interaction with MHC class I molecules. It shapes these cells' functionality and defines their response following their encounter with surrounding cells and potential target cells. NK cells expressing all the available IR (KIR in humans, Ly49 molecules in mice, and NKG2A in both species), cannot acquire efficient immunosurveillance functions, as this requires from the potential target cell a complete loss of MHC class I molecules. The wide expression of the latter molecules, up to six different in humans, requires a clonal distribution and

selectivity of the NK cell repertoire. Indeed, individual NK cells express only a subset of the available IR, generating specialised NK cells, able to detect specific and particular alterations of MHC class I molecules. As a result, the NK cell repertoire expression is of great importance in these cells' functions. Its expression and development can be influenced by epigenetic factors, molecular interactions, and can follow different developmental time-courses.

3.6.1. Epigenetic influence on NK Cell Repertoire Expression

MHC class I molecules and their receptors, more specifically the IR, are not genetically related. NK cells from both species, humans and mice, are able to express IR which do not have specific MHC class I ligands [448]. NK cell repertoire distribution may be based on an efficient, or inefficient, activation of the corresponding IR gene. From the epigenetic side, two different factors play an important role in this process of (in)efficient gene activation :

i. DNA methylation

In active genes, DNA is hypo-methylated, giving the RNA polymerase full accessibility to the corresponding genes, whereas silent genes have methylated DNA. Consistent with this, the KIR and Ly49 molecules' expression was found to be epigenetically ensured by this DNA methylation process [449-451].

ii. Role of Promoters and Transcription Factors

In T cells, B cells, and olfactory cells, only a single receptor is selected from a large repertoire, and expressed per cell [452, 453]. In contrast, NK cells present a unique case of selective transcriptional activation, and are able to express several KIR or Ly49 molecules. For instance, the transcription factor T-cell factor (TCF)-1 was found to regulate the acquisition of Ly49A receptor in a dose-dependent manner during NK cell development [454, 455]. Other epigenetic factors, like promoters, were found to have an important impact on the IR expression. In KIR and Ly49 clusters, gene activation is controlled by several promoters, including some of which are bi-directional and can act as probabilistic switches [456-458]. One of these bi-directional promoters, Pro1, was found to have a correlation between its forward activity and the murine Ly49 genes' expression [456]. A study in 2008 by Li *et al.* demonstrated that single nucleotide polymorphisms (SNP) in the KIR's transcription factor binding sites, are responsible of the bi-directional promoters' functional differences, and result in different expression frequencies of the corresponding receptors [459].

3.6.2. Ontogeny and NK Cell Repertoire Formation

During development, the Ly49 receptor expression on murine NK cells starts at low levels and progressively rises and reaches, at around eight weeks of age, its definitive levels; more than 80% of adult mice's NK cells express at least one Ly49 receptor. This cumulative Ly49 receptor expression was demonstrated by Ly49$^-$ (Ly49A$^-$C$^-$G2$^-$I$^-$), and single Ly49$^+$ NK cell transfer into congenic mice. Following this transfer, both NK cell types (negative or single positive for Ly49 receptors) were able to mature, acquire other IR, and showed the same expression levels of newly acquired receptors [460]. Other in vitro studies demonstrated that the IR acquisition occurs in a non-random order during murine NK cell development: Ly49A appears before Ly49G, which appears before Ly49I [461, 462]. Such results demonstrate that the Ly49 receptor acquisition is sequential (not occurring in a burst), and additive (not subtractive). Human NK cells also present sequential expression of their repertoire. During their development, NK cells express NKG2A and then KIR molecules at different levels, creating a NK cell repertoire with different receptor combinations [463, 464].

Different factors, mostly environmental, were found to have an important role in this acquisition process. *In vitro* experiments showed that stromal cells' MHC class I molecules influence the Ly49 expression initiation and cumulative expression [462]. Interleukins have distinct effects; while IL-15 (important in NK cell development), had no role in murine Ly49 expression initiation [465, 466], efficient NK cell receptor acquisition in humans was found to be dependent on IL-15 or IL-2, but not IL-7 [463]. On the other hand, CD94/NKG2A expression appears to be induced by IL-15 in both humans and mice [466, 467].

In extension to the sequential expression of NK cell's IR, it was proposed that during this progressive acquisition, a testing of the receptor's reactivity with the cognate MHC class I molecules could happen in parallel, leading to a termination of the corresponding receptor's expression [468-470]. Once the NK cell acquires the self-specific cognate MHC class I receptor, its interaction with the corresponding MHC class I molecule would educate NK cells and stop the expression of other IR [471]. A result of such a mechanism would be a random expression of NK cell receptor repertoire before the self-specific MHC class I receptor is acquired. This conclusion may be highly attractive, but such interactions with MHC class I molecules need to be tested.

3.6.3. MHC Class I Molecules and NK Cell Repertoire Acquisition

The two most important characteristics of an organism's NK cell pool is to ensure both self-tolerance and effector functions, two features resulting from the NK cell repertoire interaction with cognate MHC class I molecules. Although the sequential and additive expression of the NK

cell repertoire were demonstrated (*cf. chapter 3.6.2. Ontogeny and NK Cell Repertoire Formatio*n), the role of MHC class I molecules in this process was proposed, but still not tested.

In 1997, Raulet et al. suggested that the Ly49 receptors' expression is stochastic, and that "the co-expression probability of two distinct IR is equal to the product of their individual frequencies", namely the "product rule" [469]. Studies in transgenic mice for Ly49C and/or Ly49A, analysing the Ly49 receptor repertoire adaptation, showed that once an endogenous Ly49 receptor is engaged with its ligand, whether the former is self-specific or not, its acquisition is limited [472, 473]. The role of MHC class I molecules was to induce the termination of the stochastic expression of IR when the NK cell expresses a self-reactive IR [474, 475]. Studies of the dose-dependent effect of MHC class I molecules on Ly49 receptors' expression, showed that the acquisition of the specific MHC class I ligand, led to a reduced frequency of NK cells expressing more than two IR [476]. MHC class I molecules seem to control the NK cell subsets' size, through inhibitory signals, generating a population of NK cells with a specific IR repertoire capable of efficiently detecting missing-self, while being tolerant to the self. Skewing of these educated NK cell subsets, which is highly beneficial for the organism, may be due to their specific expansion, as demonstrated by their higher ability to proliferate as a response to IL-15 [476]. In C57BL/6, MHC class I deficient, and single allele MHC class I mice, the comparison of the "observed" with the "expected" (product rule) IR expression's frequencies revealed deviations, a result of both, MHC class I-dependent and independent events [477].

The effect of HLA class I molecules on NK cell functions is well established [9, 196, 423, 425, 478], but their role in NK cell repertoire formation is less clear. While some studies showed that NK cell repertoire expression is affected by HLA class I molecules [479, 480], others found it to be sequential, random and independent of these latter molecules [481]. Based on a correlation between phenotypic studies and statistical analyses, Andersson *et al.* were able to compare the observed KIR co-expression frequencies with the expected ones, and study the effect of self-HLA class I molecules on the KIR repertoire expression. Their results, in a KIR haplotype A group of individuals, show that HLA class I molecules had the same effect on self- and nonself-KIR's expression. Evidence of an HLA class I mediated control of NK cell subsets' frequencies exist, but appear to be less strong than in the mouse. Schönberg *et al.* showed that the NK cell KIR repertoire, more specifically the HLA-C specific repertoire, is shaped by the cognate HLA class I molecules [482]. This process of structural adaptation to HLA class I molecules does not seem to be in place during the neonatal period. No bias toward self-HLA class I recognition was detected in cord blood (CB) NK cells in both KIR haplotypes A/A and B/B [483]. Functionally, CB NK cells were found to degranulate less than adult NK cells, with a higher degranulation for educated NK cells compared to the non-educated ones, indicating that both mechanisms, NK cell tuning and KIR repertoire adaptation to HLA class I molecules, are uncoupled.

Results of MHC class I role in NK cell repertoire formation cannot be extrapolated between inbred mice and outbred humans. The differences observed between the two species may be induced by inbreeding, in the mice case, which presents allelic variations of both MHC class I molecules and Ly49 receptors, thus increasing the specificity and the affinity between these two molecules. The KIR/HLA and Ly49/MHC systems comparison should benefit from similar analysis platforms (controlled MHC class I expression coupled to high-dimension flow cytometry). The engagement failure of an IR with its cognate MHC class I molecule during NK cell development does not represent a death sentence for these cells. In healthy donors and in TAP-deficient patients (same as in WT and MHC class I KO mice), such NK cells are able to develop, but display reduced functional capacities [106, 484]. Such NK cells represent a very good tool to study HLA class I molecules' influence on their receptors' acquisition. In addition to all the above mentioned factors which may influence KIR acquisition, infections, more specifically viral infections, were reported to have a high impact on the NK cell repertoire formation.

3.6.4. Viral Infections and NK Cell Repertoire Expression

During virus infections, NK cells produce a fast response [485, 486], and in mouse CMV infections, it is characterised by an expansion, then a contraction by apoptosis of specific subsets [487, 488]. With other viruses, like Puumala hantavirus, the expanded NK cell subsets were found to last for more than 2 months after the infection [489]. One of these expanded populations was the highly mature $CD57^+$ $NKG2C^+$ NK cells, which reached its peak at day 5 post infection, explained by the NK cell interaction with infected target cells, known to upregulate the expression of HLA-E, the specific ligand for NKG2C. CMV, like other viruses, developed complex mechanisms aimed to evade the immune system (*cf. chapter 1.4.3.1. Viral Infections*) [231, 490-492], one of which is to keep NK cell IR engaged [253]. The above mentioned expansion of $NKG2C^+$ NK cells was also reported in CMV infected patients [493-497], or co-infected with HIV-1 [498, 499], hantavirus [489], chikungunya [500], HBV and HCV [501], suggesting that infection by CMV may shape the NK cell repertoire. Indeed, a detailed analysis of the expanded $CD57^+$ $NKG2C^+$ NK cell subset revealed a skewing toward the expression of self-KIR molecules [496, 501]. This skewing was shown to be time-stable demonstrating the dynamic side of NK cell phenotype and functions. Another unexpected result was the expansion of NKG2C-independent subsets of NK cells expressing act-KIR, suggesting a role of the latter molecules in NK cell response to CMV infection [496].

Throughout their lifespan, NK cells showed that they are able to adapt to their environment, tune their functions, and undergo specific expansions following virus infections.

Looking to these findings, one could hypotheses the different theories about HLA class I molecules and NK cell KIR repertoire formation, especially after the demonstration that the CMV infection status influences NK cell KIR-ome. Still one aspect that all the previous studies have not taken in consideration, is studying the NK cell repertoire in a HLA class I deficient (or low-expressing) environment.

3.7. Trogocytosis

NK cell functions are controlled by a balance between activating and inhibitory signals transmitted through a panel of AR and IR on their cell surface. However, since the description of the membrane and protein exchange between NK cells and their surrounding cells, namely trogocytosis, NK cell functions proved to be more complex than stated in the basic description above. This phenomenon may have high impact on NK cell function and presumes that they can react to stimuli from molecules they never expressed, but acquired from the environmental cells.

3.7.1. Characteristics and mechanism

Trogocytosis is a process in which two cells exchange membrane fragments and proteins following an interaction between a receptor, belonging to the cell 1, and its specific ligand, belonging to the cell 2. This transfer does not only imply extracellular proteins, but may also include patches of membrane as well as intracellular and trans-membrane proteins.

In 1973, Bona *et al.* were the first to describe a LPS exchange between lymphocytes [502]. This mechanism was later extended to the uptake of antigens, exosomes, nanotube formation and finally trogocytosis [503].The first demonstration of trogocytosis in NK cells was provided by two independent works which described the transfer of H2-D^d to the Ly49A^+ NK cells derived from H2-K^b genotype. Ly49A^+ NK cells, derived from C57BL/6 mice, were able to acquire H2-D^d from the surrounding cells after transfer into Balb/c mice [504], or in a transgenic mouse model expressing H2-D^d at different levels [440]. In humans, KIR^+ NK cells were also described to be able to acquire their HLA class I-specific ligands from surrounding cells [505].

Trogocytosis is not restricted to NK cells: $CD8^+$ T cells are able to take up, from APCs, peptide-MHC class I molecule complexes, a TCR-mediated capture [506-508]. MHC class II molecules were also shown to be captured by $CD4^+$ T cells [509]. Moreover, both $CD4^+$ and $CD8^+$ T lymphocytes capture CD80 from the APCs surface in a CD28-dependent manner [507, 510]. Using the B cell receptors (BCR), B cells are also able to acquire cell membrane-coupled antigens [511]. The transfer of molecules in the opposite direction, from NK cells to the donor cells, in both humans and mice, has been shown to be possible [512]. But is this uptake specific?

The first two studies describing NK cell trogocytosis demonstrated that the H2-D^d uptake by Ly49A on NK cells, was a result of the specific interaction between these two molecules. An anti-Ly49A (A1 clone) antibody, or an anti-H2-D^d antibody were able to inhibit the transfer [440, 504]. In fact, Ly49A can be identified by three different antibodies, JR9-318, YE1-48 and A1, each recognising a different epitope. The A1 clone (widely known as $Ly49A^{B6}$) recognises the epitope which binds to H2-D^d, masked when both molecules interact, whereas both epitopes recognised by the JR9-318 and YE1-48 clones are always accessible. In a similar way, the transfer of MICA and MICB was found to be dependent on their interaction with NKG2D [513, 514]. Other studies showed that the uptake of HLA-Cw6 by NK cells was found to be enhanced but not dependant on KIR2DL1 [515]. Such results do not have to contradict the former ones, as following trogocytosis, the molecule transfer may be accompanied by a membrane transfer, thus passively transferring molecules, and if it happens that these molecules are studied, the specificity of the trogocytosis is eliminated.

Following trogocytosis, different situations can be considered regarding the molecules and membrane patches transferred to the acceptor cell, but all end with the acquired protein internalisation [505]:

i. Molecules alone may be transferred, without membrane patches.

   This transfer was hypothesized following H2-D^d transfer to $Ly49A^+$ NK cells [504]. Such mechanism resembles MHC class I transfer through nanotubes formation, but the involved mechanistic and characterisation proofs are still elusive.

ii. Both molecules and membrane patches can be transferred, but not integrated in the acceptor cell membrane.

   Following trogocytosis, the transferred membrane may still be fixed to the acceptor cell due to the interaction of the donor cell's molecule with its ligand on the acceptor cell. Evidence of such cases are well illustrated following HLA-C transfer to target cells, especially to $KIR2DL1^+$ NK cells, and the transferred membrane removal after a brief acid wash [512, 515].

iii. The molecules and membrane's patches are integrated in the acceptor cell membrane after transfer.

   This would implicate the integration of the donor cell membrane patches into the acceptor cell membrane, in a way that allows the acquired molecules to function properly. There is

no demonstration for such a mechanism, but many evidence suggest that it may really occur. In the HLA-C transfer to KIR2DL1^{+} NK cells (stated above), not all the membranes were removed by the brief acid wash, indicating that these membranes are not just fixed to the acceptor cell by the molecules' interaction [512]. In another report, a signal transduction protein, H-Ras, which is attached to the inner face of the membrane, was found equally distributed in the receptor cell cytoplasm after trogocytosis [516].

3.7.2. Functional Consequences

Trogocytosis is a curious phenomenon affecting many cell types of the immune system, but what are its functional consequences? In order to answer this question, two different scenarios have to be taken in consideration, regarding the transferred molecules' nature:

i. The transferred molecule is a Ligand

In a ligand-type transfer, the acceptor cell has to endogenously express the corresponding receptor, and consequently, all the pathway machinery necessary for transducing the signal. The only function required from the transferred ligand is to keep its binding properties. The best examples for such a scenario is the transfer of MHC molecules to NK cells. Indeed, following the H2-D^{d} uptake by RNK.Ly49A NK cells (RNK-Ly49A is a rat NK cell line stably transfected with the inhibitory receptor, Ly49A), a reduced killing of YB2/0 target cells (a rat myeloma cell line) was observed compared to ones which did not acquire H2-D^{d} [504]. This functional consequence might be due to the *cis* or *trans* interactions between the ligand, H2-D^{d}, and its receptor, Ly49A. The HLA-G transfer, following its specific interaction with ILT2, affected NK cells (the acceptor cells) functions by reducing their proliferation capacities as well as their IL-2 response, abolishing their target killing functions, and transformed them into regulatory NK cells, capable of blocking other NK cells' functions [517]. These consequences were abolished after HLA-G and/or ILT2 blocking, confirming its specificity, as well as after the HLA-G donor cells' removal, confirming its reversibility. Other ligands transfer had similar consequences on NK cells, like the MICA acquisition which led to NK cell degranulation [514], and the reduction of NKG2D-dependant cytotoxicity following MICB transfer [513].

ii. The transferred molecule is a Receptor

In order to demonstrate functional consequences on the acceptor cell following trogocytosis, the transferred receptor has to retain its binding capacities, as well as its capability of transducing a signal in the acceptor cell, and lead to a measurable and

definable function. In this case of figure, the necessary molecules for the signal transduction have to be (i) provided by the acceptor cell, or (ii) transferred alongside the receptor molecule from the donor cell. In both cases, the transferred receptor has to be well encored in the acceptor cell membrane. Such transfers were observed in both directions; from target cells to NK cells, or NK cells to target cells. In a work by Vanherberghen *et al.*, Ly49A, was shown to be acquired by the surrounding cells [512]. Not only IR, but also AR have been reported to be transferred, with their adaptor molecules, to target cells, like 2B4 in association with SAP [518], or NKG2D and its adaptor molecules, DAP10 [513]. To date, no functional consequence of a receptor transfer has been reported, which might be due to different reasons: (i) the transferred receptor did not correctly anchor the acceptor cell membrane, (ii) the receptor cell does not have the necessary molecules for the signal transduction, (iii) the resulting function was not strong enough to be measurable, and a last reason not to be neglected, (iv) the experiment was not set-up to study the receptor transfer, but more the ligand transfer. One exception for the (iv)th mentioned reason is the work by Rechavi *et al.*, in 2007, which specifically studies the effect of H-Ras transfer from transfected B cells to surrounding cells. The authors highlight the enhanced proliferation, cytokine secretion, and ERK phosphorylation following the transfer [516].

As stated above, the importance of the trogocytosis phenomenon is that the transferred molecule and membrane patches may endow the acceptor cell with temporary functions of the donor cell. This phenomenon can provide a better explanation of some previous works, like the work by Elliott et al. in 2010 [429]. Once transferred to a MHC class I positive recipient, MHC class I negative (β2m KO) NK cells, may interact with the surrounding MHC class I molecules, their IR ligand, and acquire them by trogocytosis, thus becoming educated and functional. Trogocytosis in these experiments was not proven, as no MHC class I phenotype was performed on the transferred NK cells after their extraction from the recipient animal.

AIMS OF THIS STUDY

NK cells, the third largest population of the circulating lymphocytes, are involved in early defence mechanisms against tumours and virally infected cells. Their functions are controlled by a balance between activating and inhibitory signals transmitted through their AR and IR respectively. NK cell functional competence is acquired through education by the interaction of their IR with cognate MHC class I molecules, but the role of the latter molecules in their specific receptor repertoire formation is still a debate, especially for human NK cells. Having in our possession PBMC derived from TAP-deficient patients, we aimed at studying the interaction between HLA class I molecules and their receptors, and its effect on NK cell phenotype and functions.

Aim of article 1

NK cell KIR Repertoire Acquisition and Maturation are Strongly Modulated by HLA Class I Molecules.

In this paper we aimed at studying the influence of HLA class I molecules on their receptor repertoire formation and NK cell functions by:

(i) investigating the co-expression pattern of HLA class I receptors (KIR2DL2/3/DS2, KIR2DL1/DS1, KIR3DL1, NKG2A and CD8) as well as the maturity marker CD57 in seven TAP-deficient patients and a panel of HD.

(ii) studying the influence of HLA class I expression on NK cell functionality (cytotoxicity, degranulation as well as IFN-γ production) towards K562 cell line.

Several studies investigated MHC class I molecules' influence on their receptors' expression in both human and mice. Murine studies, performed on WT as well as single MHC class I expressing and MHC class I deficient NK cells, demonstrated the existing of both MHC class I dependent and independent factors regulating NK cell repertoire formation. Human NK cell repertoire studies were more complicated and performed only on cells expressing HLA class I molecules. Taking advantage of our TAP-deficient PBMC stock, we were able to perform the first direct study of HLA class I presence/absence influence on their receptors' expression on NK cells.

Aim of article 2

HLA Class I Expression and Its Effect on NK Cell Maturation and Cytotoxicity Gene Profile.

Based on the results published in our above mentioned paper, a parallel study was designed, aiming at:

(i) comparing the maturation processes, from $CD56^{bright}$ to $CD56^{dim}$, between HD and TAP-deficient patients by gene profiling.

(ii) identifying the different pathways implicated in NK cell cytotoxicity which may be affected by the presence/absence of HLA class I molecules on NK cell surface.

We previously demonstrated a strong influence of HLA class I molecules on their receptors expression as well as NK cells maturation and functionality. In this brief report, we aimed at studying by whole genome microarrays the effect of HLA class I molecules on the different pathways implicated in NK cell maturation from $CD56^{bright}$ to $CD56^{dim}$ as well as cytotoxicity following co-culture with K562 cells.

MATERIALS AND METHODS

Solutions and cell culture media.

FACS Buffer: PBS, 0.5% foetal calf serum (FCS).

Complete DMEM: DMEM medium supplemented with 100U/mL penicillin, 100µg/mL streptomycin, 2mM glutamine, 100µM 2-β-mercaptoethanol, and 10% FCS.

Complete RPMI: RPMI 1640 medium supplemented with 100U/mL penicillin, 100µg/mL streptomycin, 2mM glutamine, and 10% FCS.

Cell lines.

Human cell lines

- K562 cells (human myelogenous leukemia line) as well as EBV-immortalised B cells were cultured in complete RPMI.

  Fibroblasts: HF-ME primary fibroblasts derived from patient ME (provided by Dr Dewton Vasconcelos, Laboratory of Medical Investigation, University of São Paulo School of Medicine, Brazil), STF1-169 immortalised fibroblast cell line established from a TAP-1 deficient patient [72], and STF5-169 immortalised fibroblast cell line derived from a HD (both cell lines provided by Prof de la SALLE, University of Strasbourg, France), were all cultured in complete DMEM.

Murine cell lines

- YAC-1 cells, from a murine T-lymphoma established from a tumour induced by Moloney sarcoma virus in an A/Sn mouse, were cultured in complete DMEM.
- EL4-D^d-GFP cells, an EL4 cell line transfected by a plasmid expressing the H2-D^d molecule coupled to GFP [504], were kindly provided by Dr. Petter Höglund, Centre for Hematology and Regenerative Medicine, Karolinska Institutet, Stockholm, Sweden. EL4-D^d-GFP cells were cultured in complete RPMI.

Human subjects and blood samples.

Peripheral venous blood was obtained from patients with a confirmed TAP-deficiency and from a panel of HD, from which PBMC were isolated by Ficoll Hypaque density gradient centrifugation. All individuals included in the project gave written informed consent according to the Declaration of Helsinki. The study was approved by the National Research Ethics Committee of Luxembourg (201109/05).

Animals.

C57BL/6 (WT) were obtained from Harlan, Horst, The Netherlands, and TAP-1 KO (on the C57BL/6 background) from the Jackson Laboratories, USA. Mice were kept under specific pathogen-free conditions and used at the age of 8-10 weeks. All procedures respected current European regulations and were approved by the National Animal Research Authority.

KIR and HLA class I genotyping.

Genomic DNA was isolated either from fresh blood using a QIAamp DNA Blood Mini Kit (QIAGEN) or from EBV-transformed B-lymphoblastoid cells. Both genotypings were performed using PCR sequence–specific primers. For HLA-A, HLA-B, and HLA-C, commercial Micro SSPTM plates and kits (One Lambda, Canoga Park, CA) were used, according to the manufacturer's instructions.

Murine cell preparation.

Spleens were extracted from 8 to 10 weeks old WT and TAP1-KO mice, and passed through a 40µm sieve. Red blood cells were lysed with ACK lysing buffer (Gibco®), then splenocytes were passed through a Nylon wool column to enrich the cell suspension with NK cells. Nylon Wool Non-Adherent (NWNA) spleen cells were then used in the different experiments.

Antibodies and flow cytometry.

For phenotype studies, cells were stained with fluorochrome-conjugated monoclonal antibodies (mAb) according to the experiment set up, then measured on a FACSCanto I or a FACSAria SORP (both from BD Biosciences). At least 3.10^5 live cells were acquired per sample. Analyses were done with Kaluza® Analysis Software v1.2 (Beckman Coulter). Dead cells and doublets were excluded from the analyses. NK cells were identified as $CD14^-$ $CD19^-$ $CD3^-$ $CD56^{dim}$ in human samples, and $CD3^-$ $NK1.1^+$ in murine samples, and then the percentage of positive cells for each studied marker was determined. Co-expression was calculated using the "Boolean" and the "Tree" functions.

Functional assays (interferon-γ production and degranulation assay).

NK cell response following (i) tumour cell encounter and (ii) cross-linking with antibodies, was studied.

In **murine** NK cell studies, (i) NK cells were negatively purified from NWNA spleen cells using Miltenyi NK isolation kit according to the manufacturer's instructions, then co-cultured with YAC-1 cells for 6 hours at a ratio of 2:1 in 100µL of complete DMEM and 35U/mL of recombinant human interleukin-2 (rhIL-2) (R&D Systems, Abingdon, UK). Negative controls were performed

with purified NK cells alone. (ii) For antibody cross-linking, flat bottom 96 well plates were coated with anti-NK1.1, anti-CD137 (negative control) or anti-IgG2a (isotype control) antibodies at a concentration of 10µg/mL. NWMA spleen cells from WT and TAP1-KO mice were incubated for 6 hours at a concentration of 10^6 cells in 100µL of DMEM culture medium.

In **human** NK cell studies, a total of 10^6 PBMC was co-cultured with K562 cells at a ratio of 7:1 in 100µl complete RPMI with 1000U/mL rhIL-2 (R&D Systems, Abingdon, U.K.). Negative controls were performed with NK cells alone.

In **both** studies, for degranulation measurement, the anti-CD107a mAb was added at the beginning of the experiment. After one hour of incubation, monensin and brefeldin A (GolgiStop and GolgiPlug, respectively, both from BD Biosciences) were added for a final concentration of 2µM and 1µM respectively. After another 5 hours of incubation, NK cells were stained for surface antigens according to the experiment set up. For IFN-γ assay, cells were fixed and permeabilized using Cytofix/Cytoperm solution (BD Biosciences), and then stained with anti-IFN-γ mAb. In both cases, dead cell marker (DCM) was used to exclude dead cells.

Cell sorting.

Human PBMC were stained with the corresponding mAb, and were kept during all the steps in complete RPMI. In order to stain and exclude dead and apoptotic cells, Hoechst 33342 (Invitrogen; 100 ng/mL) was added just before measurement to stain DNA of the permeable cells. Cell sorting was performed on a FACSAria SORP (BD Biosciences). NK cells were identified and selected as described in the § Antibodies and flow cytometry.

Cytotoxicity assays.

Prior to the cytotoxicity assays, 2.10^6 PBMC, derived from TAP-deficient patients or HD, were (i) directly used after de-freezing (fresh PBMC), (ii) incubated overnight with 200U/mL of rhIL-2 (R&D Systems, Abingdon, UK) (act-ON-PBMC), or (iii) after 5 days of activation with 200U/mL of rhIL-2 (act-5d-PBMC). Both NK cells and K562 cells were sorted prior to co-culture. K562 cells were stained using a CellTrace CFSE (Molecular Probes) according to the manufacturer's instructions. Hoechst 33342 (Invitrogen; 100ng/mL) was added just before measurement. Live/$CFSE^+$ K562 cells were sorted and added to previously sorted $CD56^{dim}$ NK cells (c.f § Cell sorting) at different NK/K562 ratios and then centrifuged. The pellet was re-suspended in 100µl of complete RPMI in 96-well V-bottom plates, which were briefly centrifuged before a 5h incubation at 37°C, 5% CO_2. In parallel, live/$CFSE^+$ K562 cells were incubated alone to calculate their spontaneous death. After the incubation, the dead cell marker TO-PRO-3 (Molecular Probes) was added at a final concentration of 1mM to identify the percentage of dead K562 cells. After flow cytometry measurement, the percentage of specific death was calculated as

specific death = [(death - spontaneous death)/(100 - spontaneous death)]x100.

NK cell preparation for microarrays.

For microarrays, in order to investigate the affected pathways following NK cell activation, $CD56^{dim}$ NK cells, derived from 2 TAP-deficient patients [95] and 4 HD were sorted and co-incubated with K562 cells according to the cytotoxicity assays protocol, but at a unique ratio of 5/1 (NK/K562). After 1h and 5h of incubation, Hoechst 33342 (Invitrogen, 100ng/mL) was added, and live $CD56^{dim}$ NK cells were sorted in Trizol LS (Life Technologies Inc.). In parallel, $CD56^{dim}$ and $CD56^{bright}$ NK cells, derived from the same sample, were directly sorted (without any prior incubation with or without K562 cells) in Trizol LS in order to study the pathways implicated in their maturation from $CD56^{bright}$ to $CD56^{dim}$.

RNA extraction.

Total RNA was extracted from the sorted $CD56^{bright}$ and $CD56^{dim}$ NK cells (before and after activation) according to the standard Trizol chloroform protocol provided by the manufacturer, life technologies ®.

Microarrays.

Samples were prepared according to Affymetrix WT expression kit for Affymetrix Whole Transcript Expression Arrays (Life Technologies Inc.). RNA quality and quantity was ensured using the Bioanalyzer (Agilent, Inc) and NanoDrop (Thermo Scientific, Inc) respectively. Per RNA labeling, 80 nanograms of total RNA were used in conjunction with the Affymetrix standard protocol for the Human GeneChip 1.0 ST chips (Affymetrix Inc.).

Uptake assays.

NWNA spleen cells and EL4-D^d-GFP cells, with a maximum of 10^6 cells of each, were incubated at a ratio of 1/1 in 1 mL of complete DMEM for 30 min, 1 hour, 2 hours or 4 hours, at 37°C, 5% CO_2, or at room temperature. For blocking assays, EL4-D^d-GFP cells were incubated with a purified anti H2-D^d antibody during 30 min prior to the uptake assays. Cells were later on stained with the corresponding antibodies for flow cytometry analyses.

Statistical analysis.

Data are represented as mean of the different groups ± SD. Statistical significance of the difference in markers' expression frequencies between two different NK cell groups was determined by 2-tailed *t*-test. ANOVA and a post hoc Tukey test were used to evaluate whether KIR molecules are expressed differently in HD and TAP-deficient patients for each combination

of two or three of the following markers: CD8, CD57, and NKG2A. In figures, (****p<1.10^6, ***p<0 .001, **p<0 .01, *p<0.05 and $^{\#}p$<0.1). Graphs and statistical significance were performed using GraphPad Prism or R version 2.15.2.

ARTICLES

Article 1

NK cell KIR Repertoire Acquisition and Maturation are Strongly Modulated by HLA Class I Molecules

Sleiman M, Brons N.H.C, Kaoma T, Dogu F, Villa-Forte A, Lenoble P, Hentges F, Kotsch K, Gadola S.D, Vilches C. and Zimmer J.
The Journal of Immunology. 2014 Mar 15;192(6):2602-10

Preface related to article 1

Since the discovery of the different NK cell IR by A. and L. Moretta [415-419], NK cell IR repertoire acquisition became a subject of extensive study. Different models were developed and proposed, but the role of MHC class I molecules in their receptors' acquisition was not clearly established and is still an open debate, especially in human NK cells. While some studies found no effect of HLA class I molecules on KIR repertoire formation [481], others demonstrated, on a HLA-C repertoire, a bias toward the expression of self-specific KIR molecules on NK cell surface [482]. Both studies were undertaken on NK cells derived from HLA class I positive environments, making the direct effect of HLA class I molecules on their receptors' acquisition difficult to study.

TAP deficiency is a rare disease, with no more than 30 cases described till today [92, 105]. These patients present a defect in HLA class I molecules' surface expression due to a mutation in the TAP-1 or TAP-2 gene. Our laboratory has the chance to conserve the biggest stock of PBMC derived from these patients, giving us the possibility to study, for the first time, the direct effect of HLA class I molecules on their receptors' acquisition on NK cells. PBMC were provided by *Dr. Figen Dogu (Pediatric Allergy and Immunology Department, University Faculty of Medicine, University of Ankara, Ankara, Turkey), Dr. Alexandra Villa-Forte (Center for Vasculitis Care and Research, Rheumatologic and Immunologic Disease Department, Cleveland Clinic, Cleveland, OH, USA), Dr. Patrick Lenoble (Departement d'Ophtalmologie, Hopitaux de Mulhouse, France) and Prof. Stephan D. Gadola (Novartis, Basel, Switzerland)*. A multi-colour flow cytometry panel consisting of five different HLA class I receptors (CD8, NKG2A, KIR2DL1/DS1, KIR2DL2/3/DS2, and KIR3DL1), a maturity marker (CD57), two functionality markers (IFN-γ, CD107a), as well as the necessary ones to identify NK cells (CD14, CD19, CD3, CD56, CD16 and DCM), was designed with the help of *Nicolaas H.C. Brons (Core Facility Flow Cytometry, Public Research Center for Health, Luxembourg)*. PBMC derived from seven TAP-deficient patients and a panel of healthy donors (HD) were phenotyped and the co-expression frequencies of the different HLA

class I receptors were compared. Statistical analyses were performed by *Tony Kaoma (*Genomics Research Unit, Public Research Center for Health, Luxembourg) HLA class I and KIR genotype were performed by *Dr. Carlos Vilches (Laboratory of Immunogenetics-HLA, University Hospital Puerta de Hierro, Majadahonda, Madrid, Spain), Prof. Katja Kotsch (Surgery Department, Medical University Innsbruck, University of Innsbruck, Austria)* and *Dr. Francois Hentges (Laboratory of Immunogenetics and Allergology, Public Research Center for Health, Luxembourg).*

In six of the seven TAP-deficient patients, in comparison to the HD, an increase of KIR expression was observed on the $CD8^+$ $NKG2A^+$ NK cell subpopulation, but not on the $CD8^-$ $NKG2A^+$ subset. This increase suggests a possible novel role of CD8 in NKG2A and KIR repertoire acquisition. NK cell maturation was also investigated by combining the analysis of the expression of CD57, NKG2A and KIR molecules. TAP-deficient NK cells showed a significant accumulation of the $NKG2A^+$ KIR^+ NK cells only in the $CD57^-$ subset indicating a blockage in the NK cell maturation (reduced frequency of $CD57^+$ NK cells). These results demonstrate that in a TAP-deficiency, or HLA class I defective surface expression context, HLA class I receptors' acquisition is disturbed by the absence of their ligands. NK cells tend to up-regulate the co-expression of CD8, NKG2A and KIR molecules, and stop the acquisition of CD57 demonstrating a strong influence of HLA class I molecules on their receptors' repertoire formation as well as NK cell maturation.

Functionally, TAP-deficient NK cell hyporesponsiveness was confirmed, except for one patient (patient number 5) whose NK cells exhibited cytotoxic activity toward K562 cells, secreted IFN-γ and/or degranulated. No difference in the cytotoxic molecules' (perforin, granzyme B and granulysin), nor HLA-E expression was detected between this patient and the six others. An investigation of the HLA class I receptors' expression on functional NK cells derived from HD compared to the TAP-deficient functional outlier revealed differences in their distribution patterns. Regardless of the presence or absence of NKG2A and CD57 in TAP-deficient patient 5, NK cells that do not express KIR molecules are more functional than ones expressing a single KIR molecule.

NK Cell Killer Ig-like Receptor Repertoire Acquisition and Maturation Are Strongly Modulated by HLA Class I Molecules

Marwan Sleiman,*,† Nicolaas H. C. Brons,‡ Tony Kaoma,§ Figen Dogu,¶ Alexandra Villa-Forte,‖ Patrick Lenoble,# François Hentges,* Katja Kotsch, Stephan D. Gadola,†† Carlos Vilches,‡‡ and Jacques Zimmer***

The interaction between clonally distributed inhibitory receptors and their activating counterparts on NK cells and HLA class I molecules defines NK cell functions, but the role of HLA class I ligands in the acquisition of their receptors during NK development is still unclear. Although some studies demonstrated that HLA-C affects the expression of killer Ig-like receptors (KIR), other studies showed that NK cells acquire their KIR repertoire in a stochastic manner. Only when infected with human CMV is an expansion of self-specific KIR$^+$ NKG2C$^+$ NK cells detected. To gain more insight into this question, we compared the coexpression of different KIR molecules, NKG2A, CD8, and CD57, on NK cells in healthy donors and seven patients with deficient HLA class I expression due to mutations in one of the TAP genes. Our results show a correlation between the presence/absence of HLA class I molecules and the coexpression of their receptors. In an HLA class I low-expression context, an increase in KIR molecules' coexpression is detected on the NKG2A$^+$ CD8$^+$ subset. In functional assays, hyporesponsiveness was observed for TAP-deficient NK cells derived from four patients. In contrast, NK cells from patient five were functional, whereas CD107a$^+$ and IFN-γ^+ CD56^{dim} NK cells presented a different pattern of HLA class I receptors compared with healthy donors. Taken together, our results provide strong evidence for the role of HLA class I molecules in NK cell maturation and KIR repertoire acquisition. ***The Journal of Immunology*****, 2014, 192: 2602–2610.**

Natural killer cell functions are governed by a balance between activating and inhibitory signals transmitted through a large panel of activating receptors and inhibitory receptors (IR), respectively. Killer Ig-like receptors (KIR) bind selectively to some of the HLA-A, HLA-B, and HLA-C alleles. Furthermore, CD94/NKG2A heterodimers are specific for HLA-E, which presents peptides derived from the leader sequences of classical HLA class I molecules (1–3). These IR deliver inhibitory messages when mature NK cells meet normal autologous targets, but they also promote normal NK cell education. Only NK cells expressing at least one IR specific for self-HLA class I molecules become fully functional in this NK cell–licensing process. In contrast, in the absence of a self-HLA class I–specific IR, NK cells remain in a state of hyporesponsiveness. This mechanism allows the generation of a NK cell repertoire tuned to the optimal detection of "missing-self" (downregulation or absence of autologous HLA class I molecules) while avoiding autoreactivity (4–6).

A particular scenario for NK cell education exists in patients with genetic defects in the subunit 1 or 2 of the TAP gene. In these patients, the surface expression of HLA class I molecules is significantly reduced (1–10% of normal levels) (7, 8). NK cells from these patients were shown to be hyporesponsive ex vivo (low or absent cytotoxicity against K562 cells) (9–11). Such data support the above-mentioned models of NK cell education, in which NK cells remain "unlicensed" as a result of the strongly reduced interactions between the NK cells expressing IR and a severely HLA class I–defective environment.

An unanswered question is how NK cells acquire their clonally distributed repertoire of HLA class I binding molecules and, in particular, their KIR repertoire, because this is a distinctive phenomenon from NK cell education. An effect of HLA-C on the KIR repertoire was demonstrated by some investigators (12, 13), whereas others found the acquisition of KIR molecules to be stochastic (14, 15) except after human CMV infection, which leads to the expansion of self-specific KIR$^+$ NKG2C$^+$ NK cells (16).

In this study, we dissected the NK cell repertoire in seven TAP-deficient patients (17–20) and a panel of healthy donors (HD) by carrying out a detailed analysis of HLA class I receptors (CD8, NKG2A, KIR2DL1/DS1, KIR2DL2/3/DS2, KIR3DL1) and CD57 expression and coexpression using 14-color flow cytometry. Our results show an inverse correlation between the presence of HLA

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Received for publication October 21, 2013. Accepted for publication January 7, 2014.

This work was supported by the Luxembourgish Ministry of Research, the Fonds National de la Recherche du Luxembourg, Grant PHD-09-172 (to M.S.), and Project SAF2010-22153-C03-03 (to C.V.).

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The online version of this article contains supplemental material.

Abbreviations used in this article: HD, healthy donor; IR, inhibitory receptor; KIR, killer Ig-like receptor.

www.jimmunol.org/cgi/doi/10.4049/jimmunol.1302843

class I molecules and the coexpression of its receptors, providing new evidence in favor of a strong role for HLA class I molecules in NK cell KIR repertoire acquisition.

Materials and Methods

Human subjects and blood samples

Peripheral venous blood was obtained from seven patients with a confirmed TAP deficiency and from 18 HD (8 males, 10 females; age 25–60 y; mean age [± SD]; 36 ± 8 y). PBMC were isolated by Ficoll-Paque density gradient centrifugation. All individuals included in the project gave written informed consent according to the Declaration of Helsinki. The study was approved by the National Research Ethics Committee of Luxembourg (201109/05).

KIR and HLA class I genotyping

Genomic DNA was isolated either from fresh blood using a QIAamp DNA Blood Mini Kit (QIAGEN) or from EBV-transformed B-lymphoblastoid cells. Both genotypings were performed using PCR sequence–specific primers. For HLA-A, HLA-B, and HLA-C, commercial Micro SSPTM plates and kits (One Lambda, Canoga Park, CA) were used, according to the manufacturer's instructions.

Abs and flow cytometry

For phenotype studies, PBMC were stained for 9- or 14-color flow cytometry with fluorochrome-conjugated mAb (Table I). Stained cells were measured on a FACSAria SORP (BD Biosciences). At least 3×10^5 live cells were acquired per sample. Analyses were done with Kaluza Analysis Software v1.2 (Beckman Coulter). Dead cells and doublets were excluded from the analyses. NK cells were subsequently identified as $CD14^-$ $CD19^-$ $CD3^-$ $CD56^{dim}$, and the percentage of positive cells for each studied marker was determined. Coexpression was calculated using the "Boolean" and the "Tree" functions (Fig. 1).

Cell sorting

PBMC were stained for five-color flow cytometry sorting with the corresponding mAb (Table I). During all steps, cells were kept in cell culture medium (RPMI 1640 supplemented with 10% heat-inactivated FBS, 10 mM HEPES, 1 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin). Hoechst 33342 (Invitrogen; 100 ng/ml) was added just before measurement to stain DNA of the permeable cells (dead or late apoptotic). NK cells were identified and selected as described above.

Cytotoxicity assays

For cytotoxicity assays, both NK cells and K562 cells were sorted prior to coincubation. K562 cells were stained using a CellTrace CFSE (Molecular Probes); 10^6 K562 cells were incubated in 1 ml PBS/0.1% BSA with CFSE (5 μM) for 15 min at 37°C, 5% CO_2. The excess CFSE was quenched by addition of five volumes of ice-cold culture medium to the cells and incubated for 5 min on ice and then cells were washed and resuspended in fresh medium. Hoechst 33342 (Invitrogen; 100 ng/ml) was added just before measurement. Live/$CFSE^+$ K562 cells were sorted and added to previously sorted $CD56^{dim}$ NK cells (see *Cell sorting* section above) at different NK/K562 ratios (1:1, 5:1, and 10:1) and then centrifuged. The pellet was resuspended in 100 μl fresh medium in 96-well V-bottom plates, which were briefly centrifuged before a 5-h incubation at 37°C, 5% CO_2. In parallel, live/$CFSE^+$ K562 cells were incubated alone to calculate their spontaneous death. After the incubation, TO-PRO-3 (Molecular Probes) was added at a final concentration of 1 μM to identify the percentage of dead K562 cells. After flow cytometry measurement, the percentage of specific death was calculated as specific death = [(death − spontaneous death)/(100 − spontaneous death)] × 100.

Functional assays (IFN-γ production and degranulation assay)

A total of 10^6 PBMC was cocultured with K562 cells at a ratio of 7:1 in 100 μl cell culture medium with 1000 U/ml rIL-2 (R&D Systems, Abingdon, U.K.). Negative controls were performed with NK cells alone. For degranulation measurement, the anti-CD107a mAb was added. After 1 h of incubation, monensin and brefeldin A (GolgiStop and GolgiPlug, respectively; both from BD Biosciences) were added. After another 5 h of incubation, cells were stained with the corresponding mAb (Table I). For IFN-γ assay, cells were fixed and permeabilized using Cytofix/Cytoperm solution (BD Biosciences) and then stained with anti–IFN-γ mAb.

Statistical analysis

A two-tailed *t* test was used to assess the difference in the expression of markers by NK cells from HD and TAP-deficient patients. ANOVA and a post hoc Tukey test were used to evaluate whether KIR molecules are expressed differently in HD and TAP-deficient patients for each combination of two or all three of the following markers: CD8, CD57, and NKG2A. Graphs were constructed and statistical tests were performed using R version 2.15.2.

Results

Clinically, TAP-deficient individuals, including patients 1–4, 6, and 7 of our cohort (17–19), present a characteristic association of chronic inflammation, infection of the airways, and debilitating

Table I. Ab conjugates used for the different NK cell studies (phenotyping, sorting, or functional tests)

Usage							
Sorting	Functional	Phenotyping	Supplier	Clone	Ag	Conjugated Fluorochrome	Laser
X			Invitrogen		Dead cell marker	Hoechst 33342	UV
	X	X	Invitrogen		Dead cell marker	Blue fluorescent dye	UV
	X	X	eBioscience	61D3	CD14	Qdot 605	UV
	X		BioLegend	H4A3	CD107a	BV-421	Violet
		X	BioLegend	HCD56	CD56	BV-421	Violet
	X	X	BioLegend	HCD56	CD56	BV-510	Violet
X		X	BioLegend	OKT-3	CD3	BV-510	Violet
	X	X	BioLegend	UCHT1	CD3	BV-570	Violet
	X	X	BioLegend	RPA-T8	CD8	BV-605	Violet
	X	X	BioLegend	HCD57	CD57	FITC	Blue
X		X	BioLegend	H1B19	CD19	Alexa Fluor 488	Blue
	X	X	eBioscience	HP-MA4	KIR2DL1/DS1	PerCP-Cy5.5	Blue
		X	BioLegend	W6/32	HLA-A/B/C	PerCP-Cy5.5	Blue
		X	BioLegend	dG9	Perforin	PerCP-Cy5.5	Blue
	X		BioLegend	4S.B3	IFN-γ	PE	Yellow-green
		X	BioLegend	DH2	Granulysin	PE	Yellow-green
		X	BioLegend	3D12	HLA-E	PE	Yellow-green
	X	X	ImmunoTools	LT19	CD19	PE-DY647	Yellow-green
	X	X	Beckman Coulter	Z199	NKG2A	PE-Cy7	Yellow-green
X			BioLegend	HCD56	CD56	PE-Cy7	Yellow-green
	X	X	Beckman Coulter	GL183	KIR2DL2/3/DS2	Allophycocyanin	Red
		X	Invitrogen	FGB12	Granzyme B	Allophycocyanin	Red
X			ImmunoTools	MEM-15	CD14	Allophycocyanin	Red
	X	X	BioLegend	DX9	KIR3DL1	Alexa Fluor 700	Red
	X	X	BioLegend	3G8	CD16	Allophycocyanin-Cy7	Red

skin ulcers. Patient 5 (20) presented the same clinical features but, more recently, an increase in the severity of the lung infections and skin ulcers was observed. This was accompanied by facial lesions, eventually leading to complete destruction of the nasal cartilage. Because of the patient's severe illness, the planned bone marrow transplantation could not be performed. She then developed a generalized vasculitis with cerebral involvement and seizures. After a few months in an intensive care unit, unfortunately the patient died.

With regard to NK cell investigations, we previously described two TAP-deficient patients who expressed KIR and NKG2A at frequencies comparable to HD (9). However, the expression level of NKG2A on individual NK cells was much higher in the patients (9). In the current study, we aimed at examining the HLA class I receptor repertoires in seven TAP-deficient patients and a panel of HD using 14-color flow cytometry according to the Ab panel in Table I.

NK cells were selected among single/live cells, followed by gating on $CD14^-$ $CD19^-$ $CD3^-$ cells and then on the $CD56^{dim}$ population (Fig. 1A). These NK cells were examined for expression of different KIR (KIR2DL1/DS1, KIR2DL2/3/DS2, KIR3DL1), NKG2A, CD8, and CD57 (Fig. 1B–E), which are present on donor-dependent subgroups of NK cells (Fig. 2A). Because only very limited numbers of $CD56^{bright}$ NK cells express KIR (1), this subset was not considered further in the present work. In the phenotypic studies, patient 7 was considered an outlier compared with the others, because his NK cell subsets were similar to the HD ones.

TAP-deficient $CD56^{dim}$ NK cells exhibit an increase in their KIR molecules' coexpression, especially on the $NKG2A^+$ subset

The percentages of NK cells expressing the studied markers varied between TAP-deficient patients and HD (Fig. 2A), with the exception of NK cells expressing 0, 1, or 3 KIR, which were not different between the two groups (only two patients had a higher frequency of NK cells expressing three KIR). In this patient cohort, the most striking difference compared with HD was the very high percentage of $NKG2A^+$ cells. Conversely, we found a slightly lower frequency of KIR^- and $CD57^+$ NK cells in the patients. The percentage of $CD8^+$ NK cells also was significantly increased in the TAP-deficient individuals.

When analyzing $NKG2A^-$ and $NKG2A^+$ NK cell subsets, we observed marked differences in KIR expression between TAP-deficient patients and HD (Fig. 2B). TAP-deficient $NKG2A^-$ and $NKG2A^+$ NK cells exhibited strong variations in their KIR coexpression distribution pattern, with a much higher percentage of KIR coexpression on $NKG2A^+$ NK cells compared with $NKG2A^-$ NK cells. In contrast, the distribution pattern of KIR coexpression was very similar in $NKG2A^{+/-}$ subsets in HD, with a higher percentage of KIR^+ NK cells on the $NKG2A^-$ subset.

The comparison of KIR-expressing cells among $CD8^-$ and $CD8^+$ NK cells showed a nonrandom distribution in the patients (Fig. 2C). Indeed, KIR^+ NK cells were more frequent in the

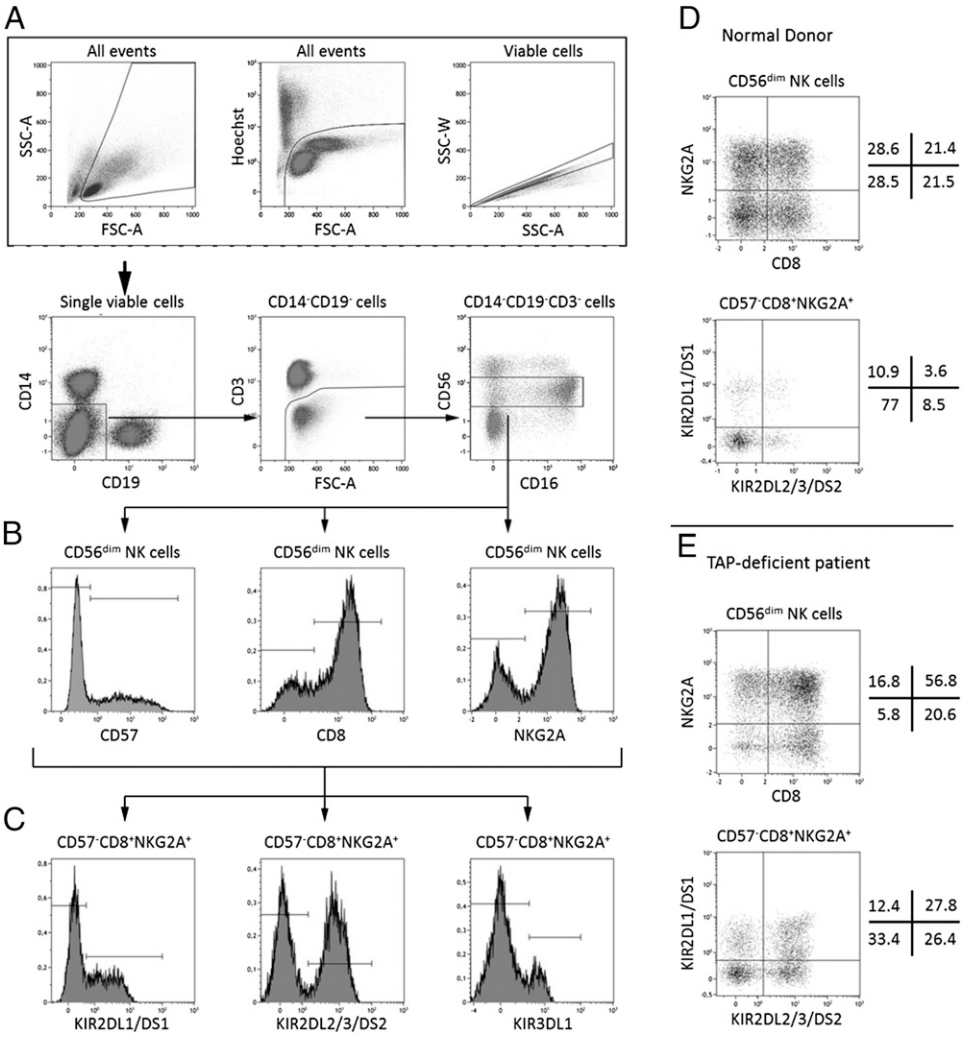

FIGURE 1. Gating strategy used to study CD57, CD8, NKG2A, and KIR coexpression on $CD56^{dim}$ NK cells. (**A**–**C**) Results of the phenotyping of a representative TAP-deficient patient. The same gating strategy was used for phenotyping the six other patients, as well as the HD. For coexpression of the different marker, the "Boolean" and "Tree" functions were applied to the gating of (B) and (C). (C) The different KIR expressions in one of the "Boolean" combinations of (B): $CD57^-$ $CD8^+$ $NKG2A^+$. Four representative dot plots showing the coexpression of NKG2A and CD8 on $CD56^{dim}$ NK cells, as well as KIR2DL2/3/DS2 and KIR2DL1/DS1 on $CD57^-$ $CD8^+$ $NKG2A^+$ NK cells derived from one HD (**D**) and one TAP-deficient patient (**E**).

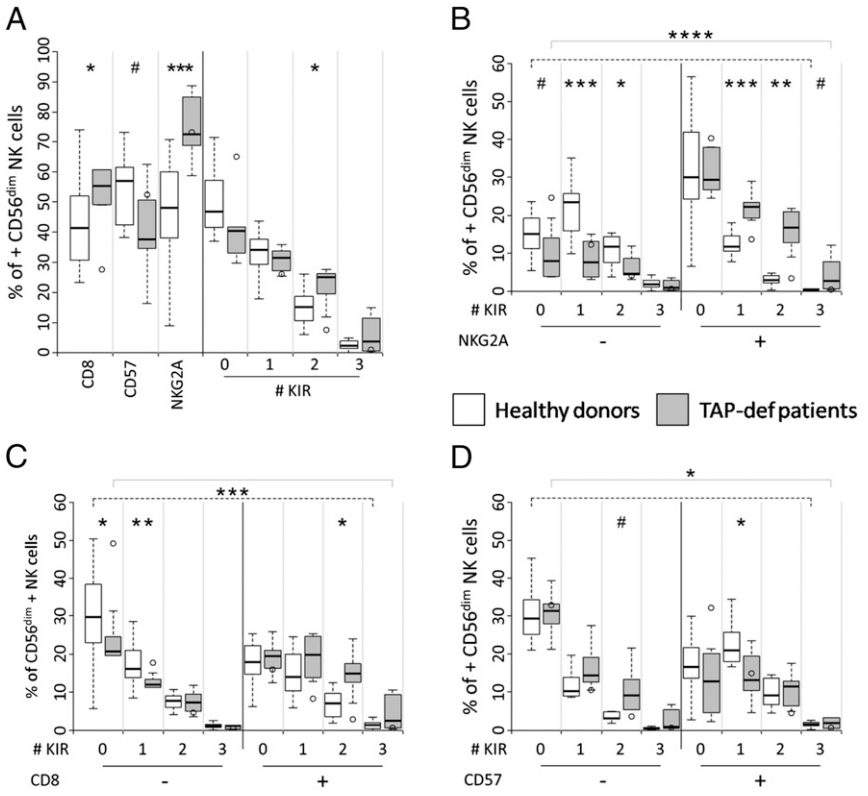

FIGURE 2. The expression of KIR molecules is influenced by the presence of NKG2A when comparing $CD56^{dim}$ NK cells from HD and TAP-deficient patients. Box plots represent the frequency of $CD56^{dim}$ NK cells expressing (**A**) or coexpressing (**B**–**D**) the studied markers in HD ($n = 9$ when CD57 is implicated; $n = 18$ when CD57 is not implicated) and six TAP-deficient patients. Circles represent TAP-deficient patient 7, who was considered an outlier. (A) By comparing NK cells from HD and TAP-deficient patients, significant differences are observed in the expression of CD8, NKG2A, and two KIR. When dissecting NK cells considering NKG2A (B), CD8 (C), or CD57 (D) expression, it appears that the presence of NKG2A and CD57 (individually) on HD NK cells has no effect on KIR distribution (horizontal dashed line). In TAP-deficient patients, KIR distribution is strongly affected by NKG2A [(B) horizontal gray line] but much less by CD57 [(D) horizontal gray line]. $^{****}p < 1 \times 10^{-6}$, $^{***}p < 0.001$, $^{**}p < 0.01$, $^{*}p < 0.05$, $^{\#}p < 0.1$.

$CD8^+$ subset than in the $CD8^-$ subset, which was not the case for the HD.

Next, we studied the influence of the maturation marker CD57 on the distribution of KIR molecules (Fig. 2D). As expected, and as previously described (21, 22), the majority of HD KIR^- cells was contained in the $CD57^-$ population, whereas the KIR^+ NK cells were better represented among the $CD57^+$ subset. Regarding the TAP-deficient NK cells, they followed the pattern of HD cells in the $CD57^-$ population. However, among the $CD57^+$ NK subset, the frequency of cells expressing one KIR was significantly lower than in HD.

HLA class I molecules strongly influence their receptors' acquisition and NK cell maturation

We compared the different combinations of these markers to explore the coinfluence of CD8 and NKG2A on KIR repertoire shaping in a HLA class I low-expression environment (Fig. 3A). The greatest difference in the percentages of KIR^+ NK cells be-

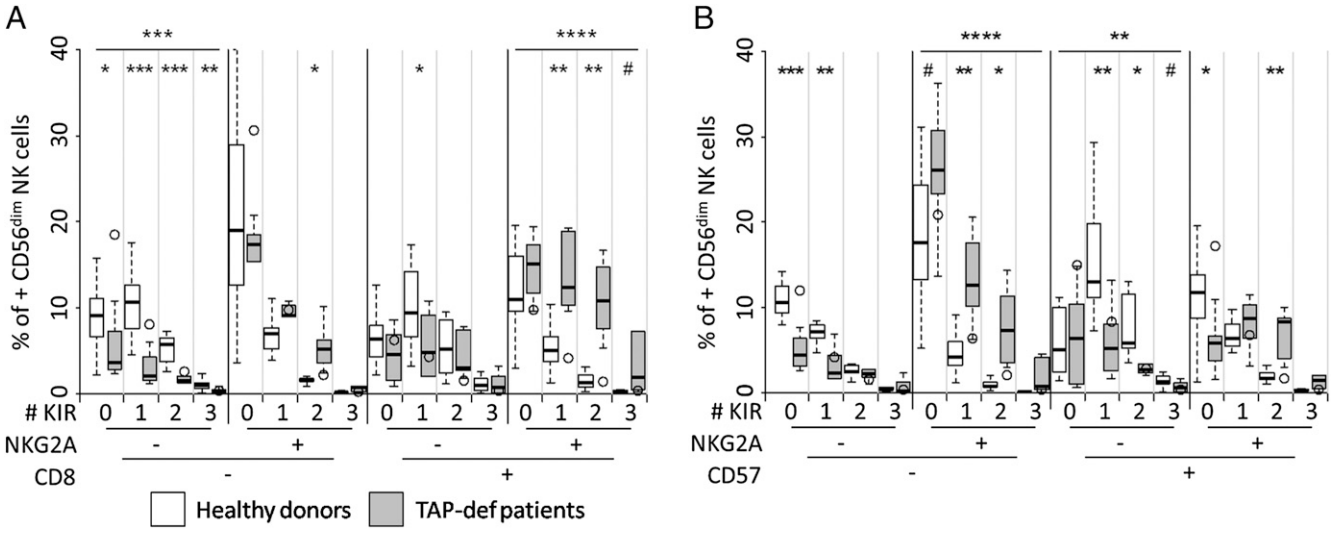

FIGURE 3. HLA class I molecules strongly influence their receptors' acquisition and NK cell maturation. Box plots represent the frequency of $CD56^{dim}$ NK cells coexpressing the studied markers in HD ($n = 9$ when CD57 is implicated; $n = 18$ when CD57 is not implicated) and six TAP-deficient patients. Circles represent TAP-deficient patient 7, who was considered an outlier. (**A**) With regard to the effect of the coexpression of CD8 and NKG2A, no significant differences were observed between HD and TAP-deficient patients for the single-positive cells ($CD8^-$ $NKG2A^+$ or $CD8^+$ $NKG2A^-$). The presence or absence of both markers together seems to affect the expression of KIR molecules on NK cells in an HLA class I low expression context. (**B**) With regard to the effect of CD57 and NKG2A on KIR distribution on $CD56^{dim}$ NK cells, significant differences were only observed for the single-positive cells for these two markers ($CD57^-$ $NKG2A^+$ and $CD57^+$ $NKG2A^-$). $^{****}p < 1 \times 10^{-6}$, $^{***}p < 0.001$, $^{**}p < 0.01$, $^{*}p < 0.05$, $^{\#}p < 0.1$.

tween the two donor groups was found in the $CD8^+$ $NKG2A^+$ and $CD8^-$ $NKG2A^-$ fractions compared with the single-positive subsets ($CD8^-$ $NKG2A^+$ and $CD8^+$ $NKG2A^-$). The major difference between KIR-expressing $NKG2A^+$ NK cells in the patients compared with HD was confirmed only in the $CD8^+$ $NKG2A^+$ subset and not among the $CD8^-$ $NKG2A^+$ cells, which is consistent with a synergistic influence of both CD8 and NKG2A on the KIR repertoire of the patients.

When combining the two markers CD57 and NKG2A (Fig. 3B), it appeared that more KIR^+ NK cells are present in the $CD57^-$ $NKG2A^+$ subset from the patients than the HD. The $CD57^-$ $NKG2A^-$ population showed the lowest amount of KIR^+ $CD56^{dim}$ NK cells. Likewise, KIR^- NK cells represented the majority in the $CD57^-$ $NKG2A^+$ subtype. When focusing on $CD57^+$ NK cells, we observed that there were more KIR^+ cells within the $NKG2A^-$ population of the HD, whereas this was not true for the patients' NK cells. KIR^+ cells were relatively rare in the $CD57^+$ $NKG2A^+$ subset in both HD and patients. Nevertheless, the HD had more KIR^- cells than the patients, with the inverse observed for $NKG2A^+$ cells expressing two KIR.

To complete the phenotypic studies, we looked at the triple combination of CD57, CD8, and NKG2A (Supplemental Fig. 1). NK cells from TAP-deficient patients and HD showed some differences, in particular among the $NKG2A^+$ cells, as well as in the $CD57^-$ $CD8^-$ $NKG2A^-$ population. A higher percentage of $CD57^-$ $CD8^+$ $NKG2A^+$ KIR^+ NK cells was observed in patients compared with HD, indicating a block of CD57 acquisition, but not for KIR molecules, in the patients.

TAP-deficient $CD56^{dim}$ NK cells exhibit hyporesponsiveness against K562 cells but express cytotoxic molecules and HLA-E

In our first study of TAP-deficient NK cells, we demonstrated that they have, in the absence of IL-2–mediated activation, no natural cytotoxic activity against the target cell line K562 or against HLA class I–deficient EBV-transformed B cell lines (no missing self recognition) (9). This result was confirmed in cytotoxicity assays using nonactivated, but purified, NK cells from four of the seven patients as effectors and K562 cells as targets (Fig. 4A). Indeed, NK cells from three patients (patients 1, 2, and 3) were not cytotoxic. Interestingly however, NK cells from one patient (patient 5) killed K562 cells to a significant degree, although not to the same extent as HD NK cells, suggesting that NK cells from this patient were able to overcome the missing education due to the lack of HLA class I molecules.

The lack of cytotoxic activity in NK cells from patients 1–3 was paralleled by the absence of a significant degranulation in the presence of K562, measured in terms of the percentages of $CD107a^+$ NK cells, which were extremely low in patients 1–4. As expected, NK cells from patient 5 degranulated upon stimulation, albeit less strongly than did NK cells from most HD. A similar hyporesponsiveness was shown for IFN-γ production by NK cells from the patients, again with the exception of patient 5 (Fig. 4B).

KIR and HLA genotyping were performed on PBMC from the seven patients (Supplemental Table I), hoping to find a unique or different genotype for the functional outlier, patient 5. KIR genotypes were identified according to Pyo et al. (23). The three possible KIR haplotype compositions were found among the seven patients (homozygous haplotype A for patient 2; heterozygous haplotypes AB for patients 1, 6, and 7; and homozygous haplotype B for patients 3, 4; and 5, Supplemental Table II). Among the three patients exhibiting haplotype B, characterized by the expression of activating KIR, only patient 5 had functional $CD56^{dim}$ NK cells. Unfortunately, such results do not explain the functional differences in NK cells from patient 5.

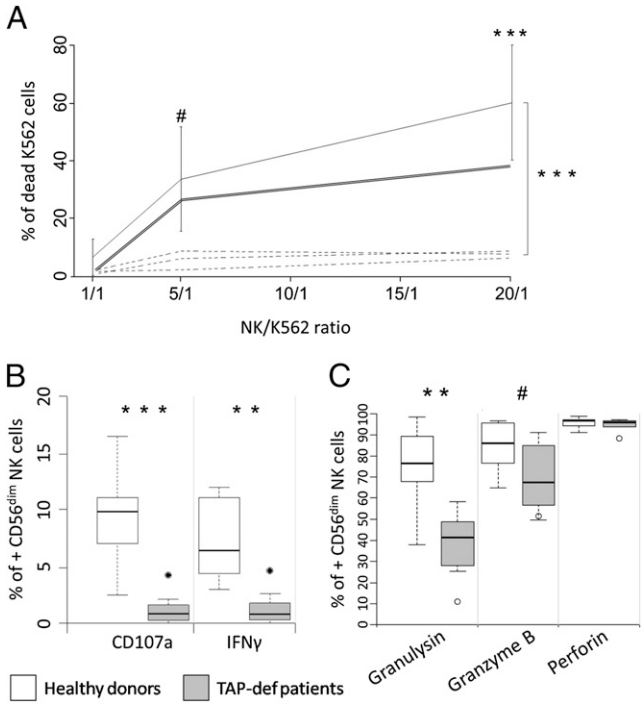

FIGURE 4. TAP-deficient $CD56^{dim}$ NK cells exhibit hyporesponsiveness toward K562 cells, although they express cytotoxic molecules. (**A**) The gray line represents HD, the dashed lines represent TAP-deficient patients 1, 2, and 3; and the bold line represents TAP-deficient patient 5. (**B** and **C**) Box plots showing the frequency of $CD56^{dim}$ NK cells derived from seven HD and six TAP-deficient patients. Outliers do not represent the same patient in (B) and (C): patient 5 is the outlier in (B) (solid dots), and patient 7 is the outlier in (C) (open dots). (A) Cytotoxicity assays were performed with purified $CD56^{dim}$ NK cells and K562 target cells incubated together for 5 h. NK cells show hyporesponsiveness for patients 1, 2, and 3 in contrast to patient 5, who presents cytotoxic $CD56^{dim}$ NK cells. (B) To confirm these results, PBMC from seven HD and five TAP-deficient patients (patients 1, 2, 3, 4, and 5) were incubated with K562 cells (ratio 7:1) and 1000 U/ml of IL-2 for 6 h. The percentage of $CD56^{dim}$ NK cells expressing CD107a or IFN-γ was determined, showing hyporesponsiveness for the studied patients with the exception of patient 5, represented as an outlier. (C) The percentage of $CD56^{dim}$ NK cells expressing granulysin, granzyme B, or perforin was determined. TAP-deficient NK cells showed no major difference compared with normal NK cells except for granulysin (circles represent patient 7, considered an outlier). $^{***}p < 0.001$, $^{**}p < 0.01$, $^{\#}p < 0.1$.

One explanation for the hyporesponsiveness of TAP-deficient NK cells could be the absence of the cytotoxic molecules contained in the granules normally released upon sensitive target cell encounter, although this would not explain the absence of IFN-γ production. Therefore, we analyzed the presence of granulysin, granzyme B, and perforin in normal and TAP-deficient NK cells (Fig. 4C). Although perforin was present in all NK cells from both cohorts, we found a slightly reduced percentage of TAP-deficient NK cells expressing granzyme B compared with HD. A more striking difference was observed for granulysin, which was present in only ~30–50% of patients' NK cells compared with 70–90% in most HD. Nevertheless, the three molecules are clearly present in TAP-deficient NK cells, so that the missing cytotoxic activity of these cells is probably not due to a lack of such factors.

The fact that most of the NK cells from the patients are $NKG2A^+$ prompted us to check for expression of HLA-E (Fig. 5A). We hypothesized that patient 5 might exhibit stronger expression of HLA-E, which would interact with NKG2A and lead to a potential education of her NK cells. The phenotyping showed that all seven patients expressed HLA-E on their NK cells, which was reduced

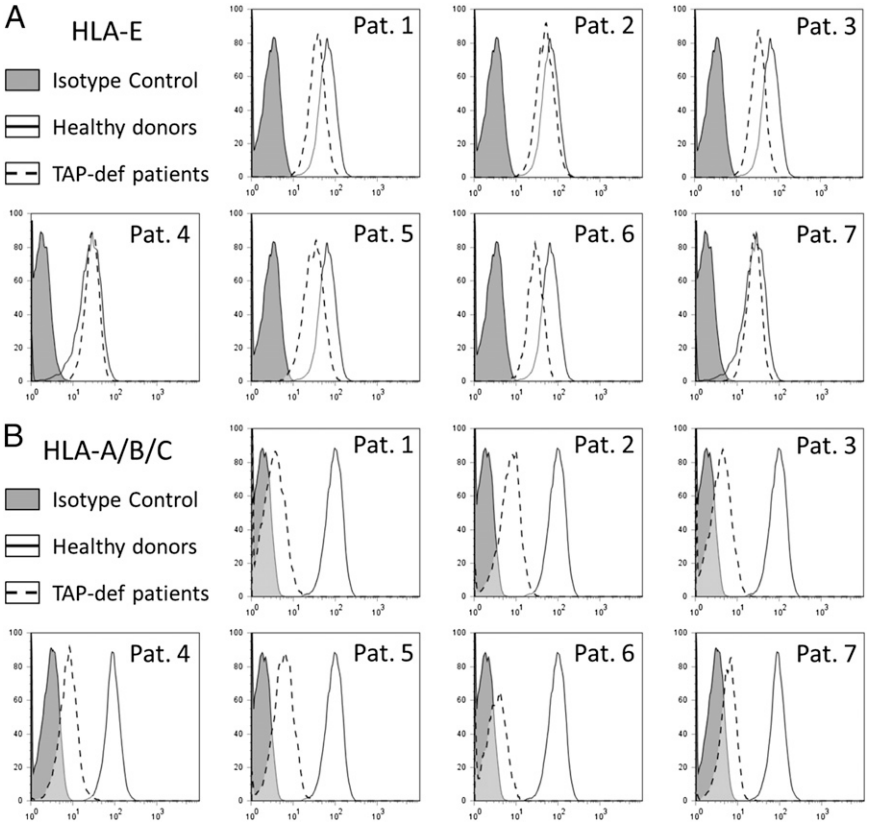

FIGURE 5. HLA-E and HLA-A, HLA-B, and HLA-C expression on HD and TAP-deficient CD56^{dim} NK cells. HLA-E (**A**) and HLA-A, HLA-B, and HLA-C (**B**) expression on CD56^{dim} NK cells for three HD and seven TAP-deficient patients. The same expression was observed for CD3$^+$, CD19$^+$, and CD14$^+$ cells.

30–50% compared with HD (50% for patient 5). In contrast, as expected and as previously shown (7–11), expression of the classical HLA class I molecules (HLA-A, HLA-B, and HLA-C) was decreased significantly in all patients (Fig. 5B), between 95 and 99% reduction compared with HD (96% for patient 5).

Different patterns for functional CD56^{dim} NK cells with regard to their HLA class I receptors in HD and TAP-deficient patient 5

We then investigated which subpopulations of NK cells from HD preferentially degranulated and/or produced IFN-γ. With regard to the patients, this analysis only made sense for patient 5 (represented as an outlier in the box plots in Fig. 6). First, we observed that most of the degranulating TAP-deficient NK cells were NKG2A$^+$ and CD8$^+$, whereas only ∼20% of the degranulating cells were CD57$^+$ (Fig. 6A). With regard to IFN-γ–producing NK cells (Fig. 5B), patient 5 presented the same pattern as HD, with the exception of CD8: 70% of IFN-γ$^+$ CD56^{dim} NK cells were positive for this marker.

Next, we analyzed the functionality, with respect to KIR expression, of NKG2A$^+$ NK cells compared with NKG2A$^-$ NK cells (Fig. 6C, 6D). A significant percentage of HD KIR$^+$ NK cells were reactive to stimulation, regardless of the presence or absence of NKG2A, but KIR$^-$ NK cells from the NKG2A$^+$ subpopulation were best represented among the functional cells. Only very few NKG2A$^-$ KIR$^+$ NK cells from patient 5 could respond to K562, whereas most of the functional cells were NKG2A$^+$. Interestingly, 20% of the IFN-γ$^+$ NK cells from the patient were NKG2A$^-$ KIR$^-$, and this percentage was twice as high as in the HD.

With respect to CD8 (Fig. 6E, 6F), the overall pattern was that, in CD8$^-$ cells, a higher number of KIR$^-$ NK cells were functional compared with KIR$^+$ cells, and this phenomenon was even more pronounced in the patient. When CD8 was expressed, NK cells without a KIR or with only one KIR were the most responsive to stimulation, with a slightly higher fraction of TAP-deficient cells compared with the cohort of HD. With regard to KIR$^-$ NK cells from the patient, the percentage of functional cells was in the same range in the presence or absence of CD8.

Cells expressing one KIR were the most functional among the CD57$^+$ population, whereas the KIR$^-$ NK cells were predominantly reactive among the less mature CD57$^-$ subset (Fig. 6G, 6H). There were more CD57$^-$ KIR$^-$ cells among the functional NK cell subset from patient 5 than all other populations, including CD57$^+$ cells.

The investigation of CD8 and NKG2A together (Fig. 7A, 7B) added little to the overall picture, but it confirmed that NKG2A$^-$ cells from patient 5 were not functional and that, in the CD8$^+$ NKG2A$^+$ population, a higher percentage of patient 5's NK cells were reactive compared with normal NK cells, with KIR absent or present.

When analyzing CD57 and NKG2A together, we found the same pattern among the CD57$^-$ NKG2A$^+$ NK cells but not the CD57$^+$ NKG2A$^+$ NK cells (Fig. 7C, 7D). No major differences between normal and TAP-deficient NK cells were noticed when three markers (CD57, CD8, NKG2A) were compared, with the exception that, again, a higher percentage of functional cells among the CD57$^-$ CD8$^+$ NKG2A$^+$ subpopulation was found in patient 5 compared with HD (Supplemental Fig. 2).

An overall conclusion is that, for TAP-deficient patient 5, functional NK cells are more represented among the CD57$^-$ CD8$^+$ NKG2A$^+$ KIR$^-$ CD56^{dim} subpopulation.

Discussion

We show in this study of a cohort of seven TAP-deficient patients that the NK cell phenotype regarding KIR and NKG2A expression is significantly different in six of the seven patients compared with a panel of HD. Our results clearly identify that HLA class I

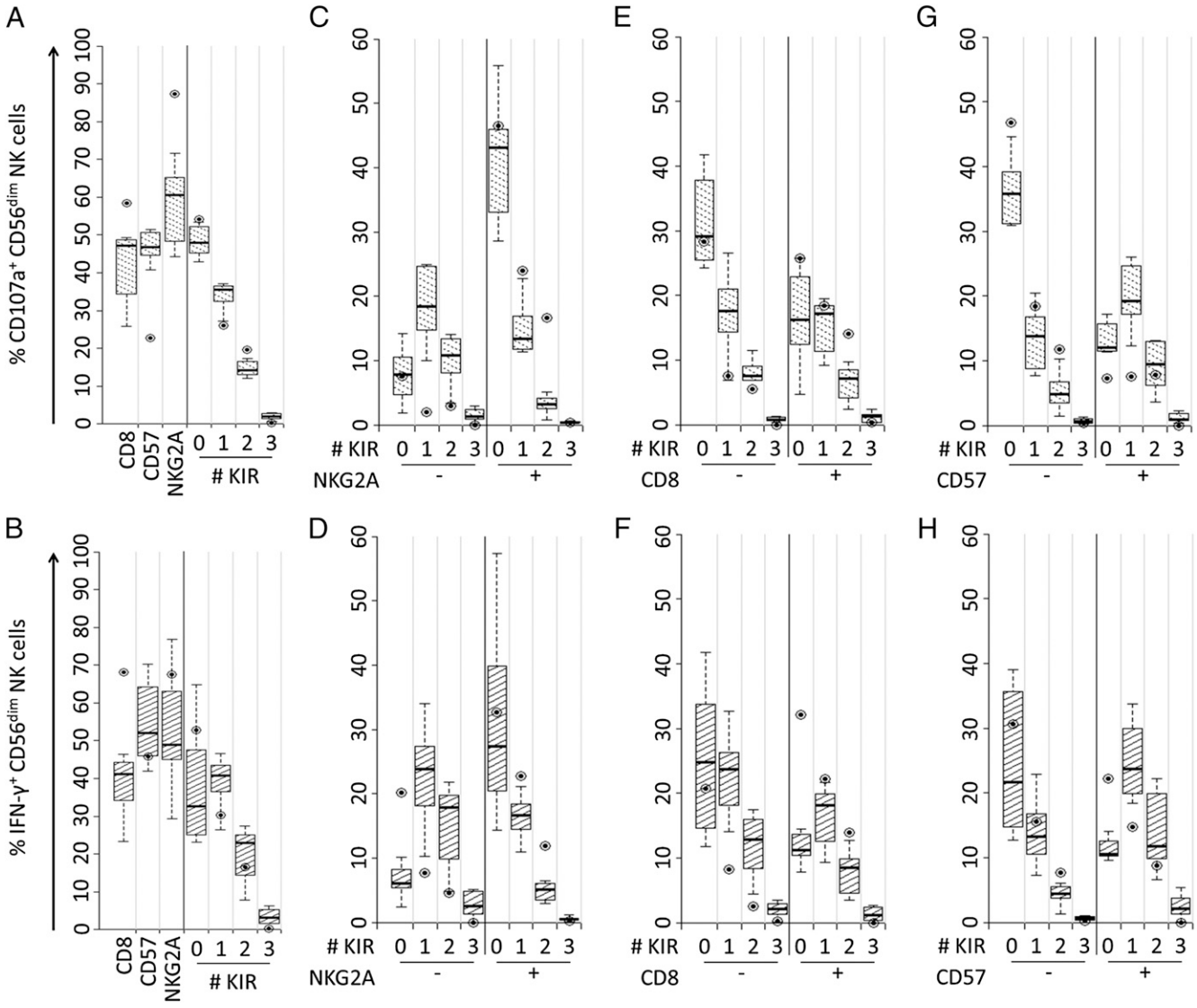

FIGURE 6. KIR molecules and NKG2A, CD8, and CD57 expression and coexpression differ in functional $CD56^{dim}$ NK cells between HD and TAP-deficient patients. Box plots represent the frequency of different markers on $CD107a^+$ and IFN-γ^+ $CD56^{dim}$ NK cells derived from seven HD. The outlier represents patient 5 (encircled black dots), who has functional $CD56^{dim}$ NK cells. When comparing NK cells from HD and TAP-deficient patient 5, differences are observed in the expression of CD8, CD57, and NKG2A on $CD107a^+$ cells (**A**) but only in the expression of CD8 on IFN-γ^+ $CD56^{dim}$ NK cells (**B**). (A and B) No differences are observed for the expression of KIR molecules. When considering the KIR distribution following the expression of NKG2A (**C**, **D**), CD8 (**E**, **F**), or CD57 (**G**, **H**) on $CD56^{dim}$ NK cells, it appears that patient 5's NK cell marker distribution on $CD107a^+$ and IFN-γ^+ $CD56^{dim}$ NK cells does not follow the same patterns as in HD. For TAP-deficient patient 5, zero KIR NK cells are always more functional than ones expressing at least one KIR, despite the presence of NKG2A or CD57.

molecules exert a strong effect on their receptors' (CD8, NKG2A, and KIR molecules) acquisition on NK cells. From a functional point of view, four of the five patients had hyporesponsive NK cells, whereas NK cells from the fifth patient were cytotoxic toward K562 and produced IFN-γ, although slightly less than the majority of HD. No major defect in TAP-deficient NK cells' cytotoxic molecules (granzyme B and perforin) was found, in contrast to a reduced expression of granulysin.

The fact that TAP-deficient NK cells are hyporesponsive was reported by us and other investigators in both human and mouse (7–11, 24). In particular, natural cytotoxic activity ex vivo was completely absent in the first two patients that we described (not included in this study) (9). According to the current models of NK cell education, only those NK cells that express at least one IR specific for self-HLA class I molecules become functional, whereas cells lacking such receptor(s) will not become licensed for cytotoxicity and cytokine production (4, 5). Following this model, NK cells from patients with TAP deficiency, in whom HLA class I expression is greatly reduced, are expected to remain hyporesponsive.

An exception to this rule is the nonclassical class I molecule HLA-E, which was reported to be subnormally expressed by TAP-deficient hematopoietic cells (25), although this was not confirmed in another patient (26). Because NK cells from one of our patients were functional, we checked for the presence of HLA-E at the cell surface and expected a higher level of this molecule on the NK cells (as representatives of lymphocytes) from patient 5. This hypothesis was based on the possibility that NK cells from this patient could have been educated through the interaction of NKG2A with its ligand HLA-E. However, HLA-E was present on the NK cells and other hematopoietic cells from the seven patients, although at reduced levels compared with HD (30–50% reduction compared with HD). Thus, if NKG2A had educated NK cells from patient 5, it would not have done so in the other

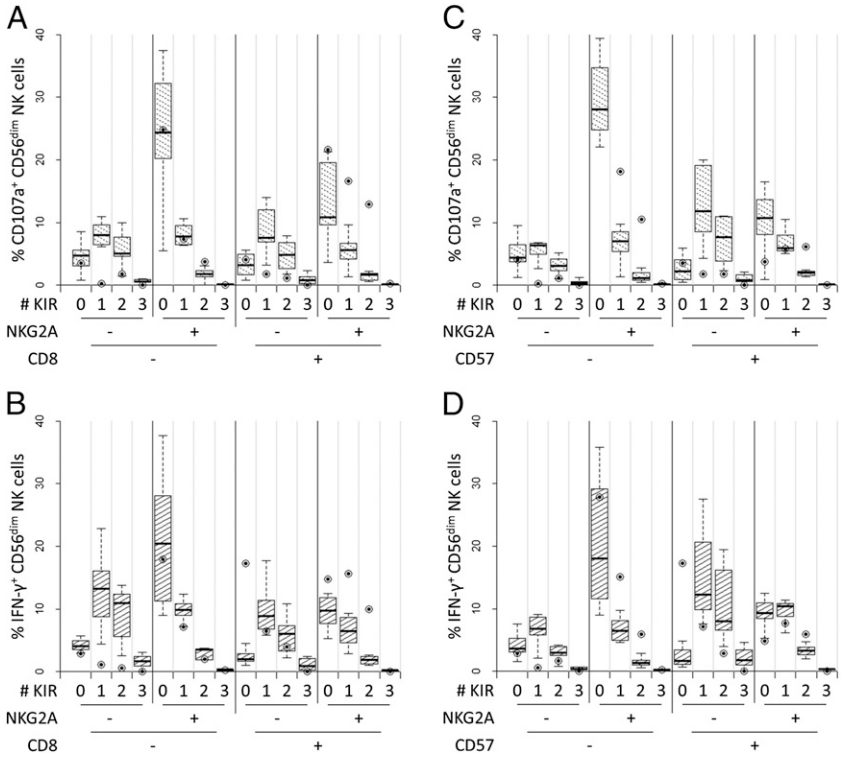

FIGURE 7. Coexpression pattern of CD57, CD8, and NKG2A on CD107a$^+$ and IFN-γ$^+$ CD56^{dim} NK cells from HD and TAP-deficient patient 5. Box plots represent the frequency of the combinations of different markers on CD107a$^+$ (**A**, **C**) and IFN-γ$^+$ (**B**, **D**) CD56^{dim} NK cells derived from seven HD. Encircled black dots represent patient 5, who has functional CD56^{dim} NK cells. Differences in the frequency of the different combinations of markers are observed between the two NK cell types. For patient 5, functional NK cells are more frequent in the NKG2A$^+$CD8$^+$ population (A, B) and the CD57$^-$ subset (C, D) compared with HD.

patients, despite comparable expression levels of its ligand HLA-E. One possible reason for the responsiveness of these NK cells is that HLA-E molecules of the patient present TAP-independent peptides, which is indeed possible (27, 28), and that the combination of HLA-E with these given peptides would allow recognition by NKG2A, whereas this would not be the case for the other patients with a different set of TAP-independent peptides. Another hypothesis is that HLA-E levels are suboptimal and still too low to educate NK cells through NKG2A, but they are not higher in patient 5 than in the hyporesponsive individuals.

A more "basic" explanation may be a strong in vivo preactivation of NK cells through HLA class I–independent mechanisms and, in particular, by inflammatory cytokines. Indeed, the patient was heavily infected at the time of blood drawing and had the most severe symptoms in our cohort, as briefly described in the Introduction.

Phenotypically, at the first level of analysis, a significantly higher proportion of patient NK cells than HD NK cells expressed NKG2A and CD8. The most striking difference was the significantly higher frequency of NKG2A$^+$ KIR$^+$ NK cells in the patients compared with the panel of HD. These results suggest that, in the absence of classical HLA class I molecules, a significant fraction of NK cells continues to "desperately" express HLA class I–specific receptors to encounter sufficient "educating" amounts of matched ligands, which can actually never occur in TAP deficiency.

Our analysis of CD57 expression revealed that KIR$^+$ NK cells were more frequent among the CD57$^+$ population and that KIR$^-$ NK cells were predominant among the CD57$^-$ subset. There were no major differences between normal and TAP-deficient NK cells in this regard. An interesting result was that, in the patients, significantly more KIR$^+$ cells were found among the CD57$^-$/NKG2A$^+$ subset than in the HD. This might suggest that a portion of the TAP-deficient NK cells acquire KIR in earlier stages of differentiation or, alternatively, that their maturation is blocked in a significant fraction of the NK cells before CD57 becomes expressed.

Because it was established that the CD8α/α homodimer, the only form of CD8 expressed by NK cells, can recognize HLA class I molecules (29, 30), we decided to integrate CD8 into the list of HLA class I ligands and to investigate its potential influence on the expression of other HLA class I ligands present in NK cells. Addison et al. (31) showed that CD8$^+$ NK cells are more cytotoxic and less prone to target cell–induced apoptosis than are their CD8$^-$ counterparts. The majority of the functional TAP-deficient NK cells in our study were CD8$^+$, which would be in favor of this claim. In contrast, CD8 by itself is not sufficient to make NK cells functional, as seen in the other patients. In any event, there is no CD8 ligand expressed on K562 target cells, so that CD8 might "only" be a phenotypic marker of the strongly cytotoxic NK cells but not functionally implicated at this level. However, it seems to play a role in the development of the KIR repertoire, at least in TAP deficiency.

With regard to the acquisition of the KIR repertoire by NK cells, two models have been proposed: the "stochastic" model (14) and the "ligand-instructed" model (13). The latter claims that, although initially stochastic, the repertoire later becomes biased toward self-specific KIR. Schönberg et al. (12) demonstrated that the "instruction" process is not yet in place in the neonatal period but appears later in life. This might explain why KIR repertoires are different among individuals, because the repertoire is shaped by the personal history of infections. However, we found a very similar KIR expression among HD NKG2A$^+$ subsets, suggesting the existence of common acquisition rules, at least for some NK subpopulations. Recently, Béziat et al. (16) showed that the bias toward self-specific KIR is characteristic of patients with a history of CMV infection but not other common herpes virus infections. This might be an explanation in many cases and would validate both the "stochastic" model (initially and in the absence of CMV infection) and the "ligand-instructed" model (bias due to CMV infection). In a humanized mouse model (mice lacking Ly49 ligands but transgenic for HLA-Cw3 and a KIR B haplotype), van Bergen et al. (32) found that, in the presence of HLA-Cw3, the

corresponding IR KIR2DL2 was reduced both in frequency and in expression level.

With regard to the expression of NKG2A and KIR in TAP deficiency, the acquisition rules are likely to be disturbed by the very low number of HLA class I molecules in the environment. Nevertheless, among NKG2A$^+$ cells, KIR molecules are present at higher levels than in HD. This could reflect that, under physiological conditions, KIR acquisition is stopped once enough "matching" IR–HLA class I interactions are attained by the individual NK cell, whereas the process continues in TAP deficiency because the limiting partner is absent. However, the reason why not all TAP-deficient NK cells express NKG2A and several KIR remains unknown. Thus, our results suggest that HLA class I molecules have a strong influence on KIR expression and point toward the existence of additional NK cell–intrinsic, HLA class I–independent factors that prevent KIR overexpression. In the context of the increasing use of NK cells in clinics, the fact that HLA class I molecules have a strong impact on the acquisition of their receptors could become important from a translational perspective.

Acknowledgments

We thank all donors, especially the patients, who volunteered to provide blood samples. We also thank Aurélie Poli for help with setting up some experiments, Elisa Cisneros for help with KIR genotyping, and Maud Thérésine, Victoria El-Khoury, and Nassera Aouali for help in performing the functional tests.

Disclosures

The authors have no financial conflicts of interest.

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Supplemental data

NK cell KIR repertoire acquisition and maturation are strongly modulated by HLA class I molecules

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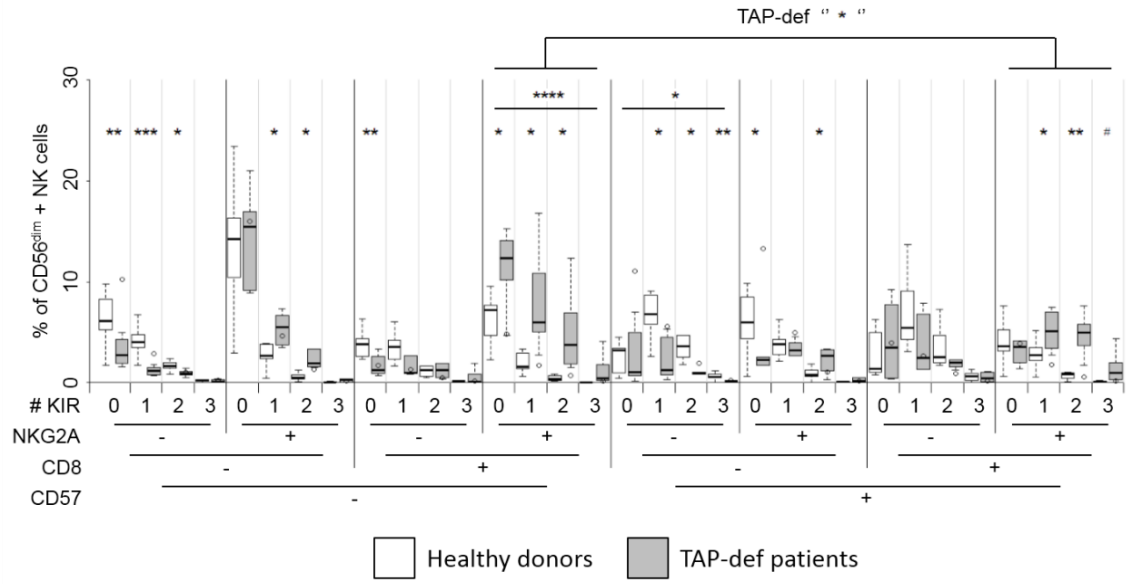
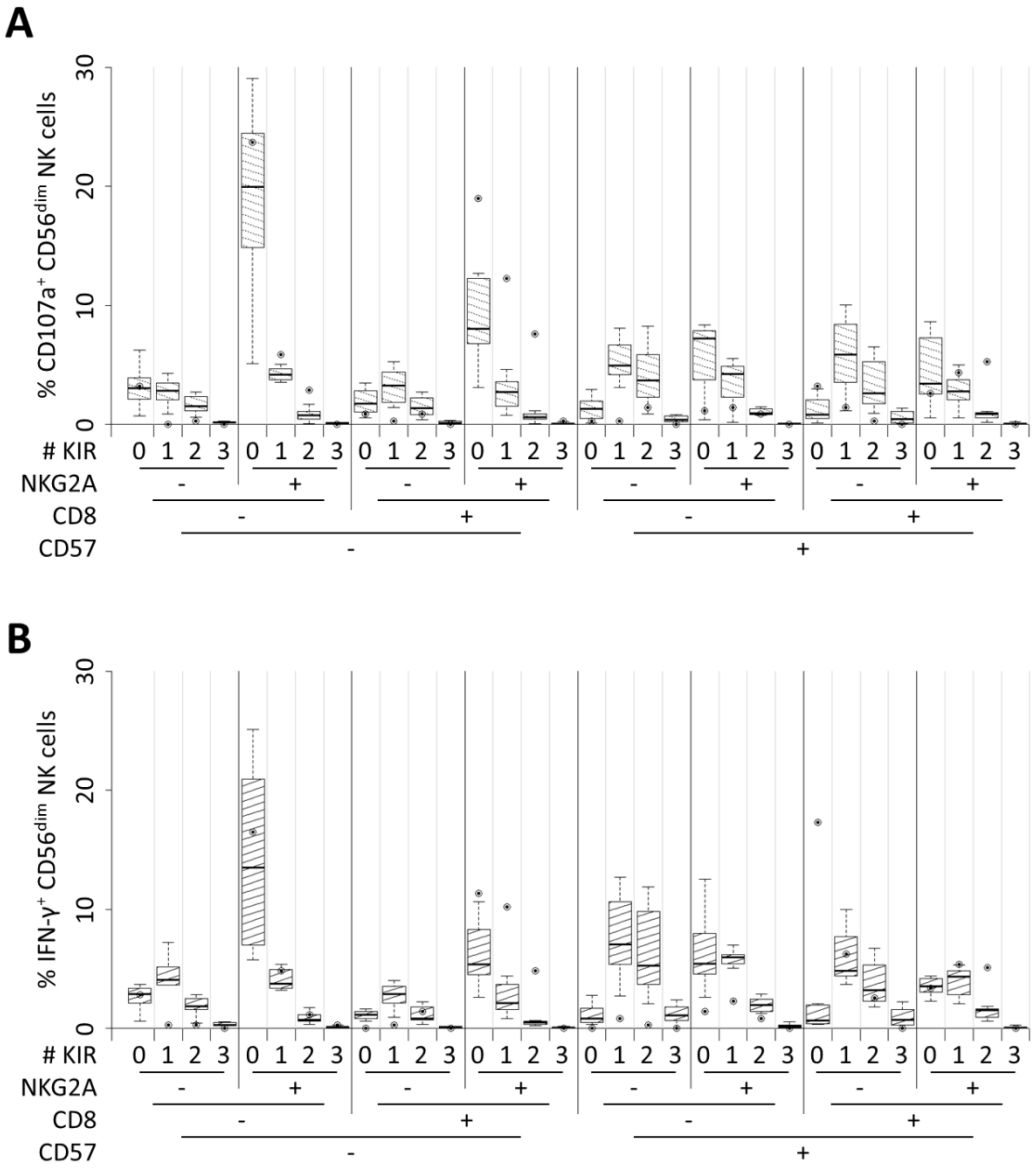
Running title: HLA class I receptors repertoire in TAP-deficiency



Supplemental Figure 1: Influence of the combination of CD57, CD8 and NKG2A on KIR molecules expression in CD56^{dim} NK cells derived from healthy donors and TAP-deficient patients.

TAP-deficient (n=7) and HD (n=9) NK cells were phenotyped according to the antibody frame (Table 1). Boxplots represent the frequency of CD56^{dim} NK cells co-expressing CD57, CD8, NKG2A and KIR molecules in nine HD (white boxes) and six TAP-deficient patients (grey boxes). The comparison of the different markers' distribution shows that CD57^{-} CD8^{+} NKG2A^{+} KIR^{+} NK cells are more present in TAP-deficient patients compared to HD, suggesting a block of CD57, but not HLA class I receptors' acquisition in the patients. Circles represent the TAP-deficient patient 7 considered as an outlier. In figures, (****P<1x10^{-6}, ***P<0 .001, **P<0 .01, *P<0 .05 and #P<0 .1).



Supplemental Figure 2: Co-expression pattern of CD8/NKG2A and CD57/NKG2A on CD107a$^+$ and IFN-γ$^+$ CD56^{dim} NK cells from healthy donors and TAP-deficient patient 5.

TAP-deficient (n=7) and HD (n=9) NK cells were phenotyped according to the antibody frame (Table 1). Boxplots represent the frequency of different markers' distribution on CD107a$^+$ (**A**, **C**) and IFN-γ$^+$ (**B**, **D**) CD56^{dim} NK cells derived from seven HD. Circles represent the TAP-deficient patient number 5 which presents functional CD56^{dim} NK cells. When combining CD8 and NKG2A or CD57 and NKG2A for the analysis of KIR molecules distribution among CD107a$^+$ (**A**) and IFN-γ$^+$ (**B**) CD56^{dim} NK cells, healthy donors and TAP-deficient patient 5 present different distribution patterns; zero KIR NK cells being more functional than one KIR NK cells in TAP-deficient patient 5 compared to HD.

Patient nb	HLA class I genotype			KIR genotype															
				2DL1	2DL2	2DL3	2DL4	2DL5	3DL1	3DL2	3DL3	2DS1	2DS2	2DS3	2DS4	2DS5	3DS1	2DP1	3DP1
1[20]	A*24	B*14	C*08	pos	-	pos	pos	pos	pos	pos	pos	pos	-	-	pos	pos	pos	pos	pos
2[20]	A*24	B*14	C*08	pos	-	pos	pos	-	pos	pos	pos	-	-	-	pos	-	-	pos	pos
3[17]	A*26:01	B*49:01	C*07:01	pos	pos	-	pos	pos	pos	pos	pos	pos	pos	pos	pos	pos	pos	pos	pos
4[17]	A*26:01	B*49:01	C*07:01	pos	pos	-	pos	pos	pos	pos	pos	pos	pos	pos	pos	pos	pos	pos	pos
5[18]	A*01	B*08	C*07	pos	pos	pos	pos	pos	-	pos	pos	pos	pos	-	-	pos	pos	pos	pos
6[19]	A*26	B*38	C*12	pos	pos	pos	pos	-	pos	pos	pos	-	pos	-	pos	-	-	pos	pos
7[19]	A*26	B*08	C*07	pos	pos	pos	pos	pos	pos	pos	pos	-	pos	pos	pos	-	-	pos	pos

Supplemental Table I: KIR and HLA class I genotype of the seven TAP-deficient patients included in this study.

KIR and HLA class I genotypes of the 7 TAP-deficient patients were determined by SSP-PCR (see section “Materials and Methods”, “KIR and HLA class I genotyping” §) showing that they are all homozygous for HLA Class I molecule genes.

Patient nb	Deduced centromeric KIR haplotypes[¶]	Deduced telomeric KIR haplotypes[¶]	Summary
1[(20)]	3DL3-2DL3-2DP1-2DL1-3DP1- (cA01) 3DL3-2DL3-2DP1-2DL1-3DP1- (cA01)	-2DL4-3DL1-2DS4-3DL2 (tA01) -2DL4-3DS1-2DL5-2DS5-2DS1-3DL2 (tB01)	AB
2[(20)]	3DL3-2DL3-2DP1-2DL1-3DP1- (cA01) 3DL3-2DL3-2DP1-2DL1-3DP1- (cA01)	-2DL4-3DL1-2DS4-3DL2 (tA01) -2DL4-3DL1-2DS4-3DL2 (tA01)	AA
3[(17)]	3DL3-2DS2-2DL2-2DL5-2DS3-2DP1-2DL1-3DP1- (cB01) 3DL3-2DS2-2DL2-3DP1- (cB02)	-2DL4-3DL1-2DS4-3DL2 (tA01) -2DL4-3DS1-2DL5-2DS5-2DS1-3DL2 (tB01)	BB
4[(17)]	3DL3-2DS2-2DL2-2DL5-2DS3-2DP1-2DL1-3DP1- (cB01) 3DL3-2DS2-2DL2-3DP1- (cB02)	-2DL4-3DL1-2DS4-3DL2 (tA01) -2DL4-3DS1-2DL5-2DS5-2DS1-3DL2 (tB001)	BB
5[(18)]	3DL3-2DL3-2DP1-2DL1-3DP1- (cA01) 3DL3-2DS2-2DL2-3DP1- (cB02)	-2DL4-3DS1-2DL5-2DS5-2DS1-3DL2 (tB01) -2DL4-3DS1-2DL5-2DS5-2DS1-3DL2 (tB01)	BB
6[(19)]	3DL3-2DL3-2DP1-2DL1-3DP1- (cA01) 3DL3-2DS2-2DL2-3DP1- (cB02)	-2DL4-3DL1-2DS4-3DL2 (tA01) -2DL4-3DL1-2DS4-3DL2 (tA01)	AB
7[(19)]	3DL3-2DL3-2DP1-2DL1-3DP1- (cA01) 3DL3-2DS2-2DL2-2DL5-2DS3-2DP1-2DL1-3DP1- (cB01)	-2DL4-3DL1-2DS4-3DL2 (tA01) -2DL4-3DL1-2DS4-3DL2 (tA01)	AB

Supplemental Table II: TAP-deficient patients' KIR haplotype analysis.

Using KIR genotypes (Supplemental Table I), the corresponding haplotypes were determined according to Pyo et *al.* (23) showing that the three KIR haplotypes (AA, AB and BB) are found among the seven TAP-deficient patients. In table, [¶]cA01, cB01, tA01, etc stand for the centromeric (c) and the telomeric (t) segments of common KIR A- and B-haplotypes, respectively.

Discussion related to article 1

NK cell IR specific interaction with MHC class I molecules is essential for these cells' functions, making the IR repertoire formation of great interest for NK cell studies. Works on mice IR repertoire, by D. Raulet and others, led to the proposal of a stochastic sequential acquisition model. Ly49A is the first murine IR to be expressed, followed by Ly49G then Ly49I, creating the different IR repertoire combinations present in the NK cell pool [461, 462]. MHC class I molecules' role in this process was suggested to be responsible of the NK cell reactivity testing and the repertoire acquisition termination. Later studies on MHC class I-deficient or single allele expressing mice, based on the comparison of "observed" *versus* "expected" IR frequencies, demonstrated the existence of MHC class I-dependent and MHC class I-independent factors regulating the NK cell repertoire formation [477].

Human NK cell KIR repertoire formation studies proved to be more complex. Statistical and phenotypical analyses by Andersson *et al.* demonstrated an absence of HLA class I molecules' effect on KIR repertoire formation in a HLA-C-specific setting. NK cell KIR molecules are acquired stochastically and not affected by cognate HLA class I molecules [481]. On the contrary, Schönberg *et al.* showed that the NK cell repertoire formation is shaped by the cognate HLA class I molecules. A bias toward self-specific KIR molecule expression was observed in adult NK cells [482], but not during the neonatal period. NK cell KIR repertoire is initially acquired stochastically, then adapted to the cognate HLA class I molecules [483]. The differences between the above mentioned studies were later on explained by Beziat *et al.* works in which they underlined the effect of CMV infections on the KIR repertoire adaptation [495, 496, 501]. An expansion of $NKG2C^+$ self-specific KIR^+ NK cell population was observed in CMV infected individuals. These results may explain the NK cell KIR repertoire bias toward cognate HLA class I molecules described in Schönberg *et al.* studies (where no information was presented concerning the CMV status of the studied subjects).

All the available studies on human NK cell repertoire formation were performed in a HLA class I positive context, so the direct effect of the presence/absence of these molecules on their specific receptors' expression could not be studied. Our work presents, for the first time, a direct correlation between the HLA class I status and their receptors' repertoire expression on NK cells. An in-depth phenotypic study of TAP-deficient NK cells showed an increase in their NKG2A, KIR2DL1/DS1, KIR2DL2/3/DS2, and KIR3DL1 molecules' co-expression on the $CD8^+$ but not the $CD8^-$ NK cell subset. Although CD8 function on T cells is well established (its expression is essential for T cell receptor (TCR) recognition of MHC class I molecules), its expression on NK cells is not well studied. NK cell CD8α/α homodimers were found to recognise HLA class I molecules [519], and $CD8^+$ NK cells were previously shown to be more cytotoxic and less prone

to apoptosis than their $CD8^-$ counterparts [355, 356]. Our results present a novel effect of CD8 on HLA class I receptors' expression on NK cells, and indicate that in a HLA class I-defective surface expression context, CD8 can synergise with NKG2A and have a strong effect on KIR repertoire formation.

Different NK cell markers were attributed to NK cell maturation, including CD57 which was found to correlate with a unidirectional differentiation process leading to highly mature NK cells [361]. In this maturation/differentiation process, NK cells tend to lose NKG2A and gain KIR expression, a phenomenon uncoupled to their education [362]. The study of CD57 in addition to NKG2A and KIR molecules in TAP-deficient NK cells, showed a blockage in the NK cell maturation process. TAP-deficient NK cells show an increase of NKG2A and KIR molecules' co-expression on the $CD57^-$ NK cell subset indicating that the NK cell differentiation process is affected by the defective surface expression of HLA class I molecules. NKG2A continues to be expressed during (and after) KIR molecule acquisition, contrary to CD57 acquisition which seems to be blocked.

Different studies have demonstrated the hyporesponsiveness of human and mouse TAP-deficient NK cells toward tumour cells [92, 105, 106, 109, 520, 521]. In our study, this hyporesponsiveness (after 5 hours of contact with K562 cells) was confirmed for three patients in cytotoxicity assays, and in four patients (the last three plus one) who did not produce IFN-γ nor degranulated (no CD107a expression). In contrast, patient number 5 exhibited cytotoxic NK cells, which were able to secrete IFN-γ and/or degranulate following their encounter with K562 cells. KIR haplotype analyses were not able to explain this TAP-deficient functional outlier as it was not the only representative of the B/B KIR haplotype among the seven patients. Another explanation was also eliminated as all the patients expressed cytotoxic molecules: perforin and granzyme B levels were similar to HD, only granulysin levels were lower (40% for TAP-deficient patients compared with 80% for HD), but that did not seem to affect the functionality of patient 5 NK cells.

NK cell responsiveness and functionality are a result of their education by MHC class I molecules. Educated functional NK cells have to express at least one IR specific for cognate MHC class I molecules. Following this hypothesis, TAP-deficient NK cells are expected to be hyporesponsive, which was demonstrated in our work and by others before us [92, 105, 106, 109, 520, 521]. The fact that the frequency of $NKG2A^+$ NK cells was significantly higher in the patients compared to the HD, led us to check the expression of its specific ligand, the non-classical HLA class Ib molecule, HLA-E. The latter is known to present on the cell surface, peptides resulting from the leader sequences of HLA class Ia molecules (HLA-A, -B, and -C) [19], and to bind the NKG2 molecules (NKG2A, B and NKG2C) [199]. The expression of HLA-E in TAP-deficient patients is still a debate. While Furukawa *et al.* reported an expression comparable to HD [522],

Matamoros *et al.* found it to be highly reduced on PBMC derived from another TAP-deficient patient [100]. In our cohort, HLA-E was present on the lymphocytes of all the patients, but its expression was reduced from 30% to 50% compared to the level in HD. If the interaction of NKG2A with its ligand HLA-E could educate NK cells from patient 5, it would have been possible for the other patients too, especially that the functional outlier had the lowest HLA-E surface expression level, 50% reduction compared to HD.

Another plausible explanation of the exceptional NK cell functionality of TAP-deficient patient 5 would be the peptides presented by HLA-E molecules. Such peptides can be TAP-dependent or TAP-independent and may differ between the individuals [523, 524]. Our functional outlier's NK cells may have their HLA-E coupled to a peptide conferring them educational characteristics, thus making them functional. Only a peptide elution test would give us the answer to this question, but the patient unfortunately passed away after severe lung infections and a generalised vasculitis with cerebral involvement and seizures. Considering the heavy infections and health conditions at the time of the blood sampling, an *in vivo* activation of NK cells by secreted cytokines like IL-15 and IL-2 is possible, and could be the explanation for her functionality and severe condition.

The exceptional functionality of TAP-deficient patient 5 NK cells led us to investigate the HLA class I receptors' expression pattern on IFN-γ^+ or CD107a$^+$ NK cells. HD functional NK cells exhibited a normal pattern of KIR and NKG2A expression: NK cells expressing 1 KIR molecule were among the most functional ones in the absence of NKG2A or presence of CD57, whereas NK cells with no KIR molecules on their surface were among the functional cells in the presence of NKG2A. TAP-deficient patient 5 functional NK cells did not follow the same pattern: in the presence or absence of NKG2A and/or CD57, NK cells expressing no KIR molecules were among the most functional ones. Such a pattern could not be a result of education by HLA-E or the low expressed HLA class I molecules. In order to check if the possible *in vivo* IL-2 activation could lead to such results, TAP-deficient NK cells derived from patient 1 and 2 were activated for 12 hours with 200U/mL of rhIL-2 then co-incubated with K562 cells according to the same conditions in [525]. After 5 hours of co-incubation with K562 cells no IFN-γ production was observed but 15% of activated TAP-deficient CD56^{dim} NK cells degranulated (data not shown). Their HLA class I receptors' expressing pattern was different from the HD and the TAP-deficient patient 5. These results may indicate that our functional outlier's NK cells are not activated by *in vivo* secretion of IL-2. Further analyses of the different IL present in the patient's blood may have given us more insight to her unique NK cell functionality, but unfortunately this patient's materials are no longer available.

According to the different NK cell education models, the interaction of a self-specific IR with cognate MHC class I molecules educates NK cells giving them their full functionality. In accordance to this process as well as our presented results, it would be tempting to suspect a role of the inhibitory signals in NK cell KIR repertoire formation. Once a self-specific IR is acquired, its interaction with cognate MHC class I molecules (in *cis* or *trans*) would lead to an inhibitory signal educating NK cells, but also stopping the acquisition of other IR and down-regulating NKG2A. In a HLA class I-defective expression context, the expression of self-specific IR would lead to none of the above mentioned processes, as their interaction with the cognate HLA class I molecules can never occur. No inhibitory signal can be transmitted, leading to an accumulation of HLA class I receptors especially in the $CD57^-$ NK cell subset, a sign of a blockage of NK cell maturation.

Article 2

HLA Class I Expression and Its Effect on NK Cell Maturation and Cytotoxicity Gene Profile

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* Contributed equally
In preparation

Preface

Our paper dealing with HLA class I molecules' effect on NK cell KIR repertoire acquisition [525] demonstrated an overall hyporesponsiveness of TAP-deficient NK cells toward the K562 cell line, as well as a blockage in their maturation process demonstrated by an accumulation of $CD8^+$ $NKG2A^+$ KIR^+ NK cells in the $CD57^-$ subset. In this brief report we aimed at determining the different pathways, affected by the very low surface expression of HLA class I molecules, which are implicated in (i) NK cell maturation from $CD56^{bright}$ to $CD56^{dim}$, and (ii) NK cell response toward tumour cells, more specifically the K562 cell line. In both parts, (i) and (ii), two TAP-deficient patients (patient 1 and 2 according to Sleiman *et al.* [525], provided by *Dr. Patrick Lenoble, Departement d'Ophtalmologie, Hopitaux de Mulhouse, France*) and four HD were chosen, from whom $CD56^{bright}$ and/or $CD56^{dim}$ NK cells were sorted (by *Nicolaas H.C. Brons, Core Facility Flow Cytometry, Public Research Center for Health, Luxembourg* and *myself*) and then whole genome microarrays or functional tests followed by whole genome microarrays were performed by *François Bernardin (Genomics Research Unit, Public Research Center for Health, Luxembourg)*. Statistical analyses were performed by *Tony Kaoma (Genomics Research Unit, Public Research Center for Health, Luxembourg).*

In the present version of the brief report, we will quickly expose analyses and discussion of the results as work is still ongoing to better understand the study's outcome.

Results and discussion

Human NK cells can be sub-divided into two main populations according to their surface expression of CD56: $CD56^{bright}$ and $CD56^{dim}$. These two NK cell sub-populations were shown to be different phenotypically and functionally [6, 119, 336, 377], and several studies have demonstrated that $CD56^{bright}$ NK cells are the progenitors of the $CD56^{dim}$ subset [362, 378-381]. From the transcriptomic side, studies by Hanna *et al.* revealed differences in the gene expression

profile between $CD56^{bright}$ $CD16^{-}$ and $CD56^{dim}$ $CD16^{+}$ NK cells derived from a panel of healthy donors [526]. Having demonstrated a maturation blockage in TAP-deficient NK cells due to the defective surface expression of HLA class I molecules [525], we aimed in this work at examining the different pathways implicated in this maturation process in both statuses (HD and TAP-deficient) as well as NK cell cytotoxicity.

The individual comparison of each NK cell subset ($CD56^{bright}$ or $CD56^{dim}$) derived from the two statuses (HD *vs* TAP-deficient) showed considerably high numbers of differentially expressed genes (1730 and 1395 respectively) which encode mainly transcription factors and phenotypic markers (Fig. 1). When comparing the gene expression profile between the two NK cell subsets ($CD56^{bright}$ *vs* $CD56^{dim}$) in the same status (HD or TAP-deficient), only 700 and 638 genes respectively were found to be differentially expressed. Cluster analysis of these genes did not reveal any significant difference between the two cell types [(HD $CD56^{bright}$ *vs* $CD56^{dim}$) *vs* (TAP-deficient $CD56^{bright}$ *vs* $CD56^{dim}$)]. TAP-deficient and HD NK cells seem to follow the same paths while maturating form $CD56^{bright}$ to $CD56^{dim}$. The defective expression of HLA class I molecules seems to affect the $CD56^{bright}$ and $CD56^{dim}$ NK cell gene expression, but not their maturation process, at least from the transcriptomic side.

In a previous work, TAP-deficient NK cell hyporesponsiveness was broken after 11 days of activation with rhIL-2 allowing them to efficiently kill K562, DAUDI and RAJI cells, and perform ADCC [106]. Similar results were observed for the two TAP-deficient patients investigated in this study. Initially hyporesponsive (Fig. 2A), after 12 hours of activation with 200U/mL of rh-IL2, NK cells sorted from the two TAP-deficient patients were able to kill K562 cells (Fig. 2B) as well as degranulate (15% of $CD107a^{+}$ NK cells for both patients), but no IFN-γ production was detected (data not shown). This activity increased considerably after 5 days of activation (200U/mL of rhIL-2) and exceeded a 90% killing of K562 cells (Fig. 2C). TAP-deficient NK cells express normal levels of perforin, granzyme B, but reduced levels of granulysin [525]. Activation with rhIL-2 broke these cells' hyporesponsiveness, demonstrating that they have the necessary machinery to kill tumour cells but are not able to trigger it in normal conditions.

The second part of this study aimed at examining the different genes and pathways implicated in the NK cell cytotoxicity and which are affected by the defective surface expression of HLA class I molecules. To do so, $CD56^{dim}$ NK cells derived from HD and the two TAP-deficient patients were sorted, incubated with K562 cells at a ratio of 5/1 (NK/K562), then resorted for whole genome microarrays. The incubation times were chosen in order to study the early induced genes (1 hour) and the late induced genes (5 hours) resulting from K562 co-incubation. Considering TAP-deficient NK cell hyporesponsiveness, as well as the differences in the expressed gene profiles at t=0h (Fig. 1), we expected to have completely different profiles at 1 hour and 5 hours of incubation.

Three different methods were used in analysing the gene profiles generated in this study: (i) static data set comparison, when considering each NK cell subset in the two statuses (ex. $CD56^{dim}$ HD *vs* TAP-def, blue in Fig. 3), (ii) time-series expression data set, when comparing in the same status two different time points (ex. HD 1h *vs* 5h, red in Fig. 3), and (iii) comparative-time-series expression data set, when comparing two different time points in the two statuses (ex. [HD 1h *vs* 5h] *vs* [TAP 1h *vs* 5h], mauve in Fig. 3).

The gene expression differences between HD and TAP-deficient $CD56^{dim}$ NK cells was already demonstrated in the paper's first part. A static data set comparison after 1 hour of incubation showed less differences between the two statuses: only 352 genes (belonging to 6 different functional clusters) are differentially expressed, compared to 1395 genes and 22 functional clusters before tumour co-incubation (Fig. 3A, B). Following 5 hours of co-incubation, an identical gene profile is observed for the two NK cell types. TAP-deficient and HD NK cells are similarly induced although they are functionally different. Such unexpected results led us to consider a different approach: time-series expression data set analysis combined to a comparative-time-series expression data set analysis. Although "HD 0h *vs* 1h" profile showed significantly higher differentially expressed genes compared to "TAP-deficient 0h *vs* 1h" (1252 and 66 genes respectively), when performing a comparative-time-series expression data set analysis, only 12 genes (with no specific functional cluster) were found. Similar results were found for the two other comparative-time-series expression data set analysis.

The phenotypic differences between HD and TAP-deficient NK cells, like: the absence of IFN-γ production in TAP-deficient NK cells and the increase of CD69 expression in HD NK cells after 5 hours of K562 co-incubation, as well as all the cytotoxicity pathways, are not reflected in the gene profile. Such results were not expected in the light of a complete hyporesponsiveness of TAP-deficient compared to a normal responsiveness of HD NK cells toward the tumour cell line K562. Different hypotheses can be drawn in order to explain these results, but the most plausible one would be that the necessary genes in cytotoxicity pathways were not expressed at the right time in TAP-deficient NK cells. IFN-γ mRNA, as well as other molecules produced upon tumour cell encounter, were detected in TAP-deficient NK cells, but their corresponding proteins were absent. Increasing the studied time-points between 0 hour and 1 hour of incubation, coupled to a protein-array-analysis, could complete the whole-genome-microarrays performed in this study, but the availability of the biological material (TAP-deficient PBMC) is the limiting factor. In order to circumvent this problem, another analysis approach is being performed. The differentially expressed genes in Fig. 3, can be subdivided into three different clusters: (i) genes which have similar expression levels between the two statuses at t=0h, and are similarly affected by tumour co-incubation, (ii) genes which are different between both statuses at t=0h and do not change in expression by tumour co-incubation time, and (iii), the most interesting cluster; the genes which

are initially different between the two statuses and are affected by the tumour co-incubation. These last genes are not numerous, but are under detailed analyses for the time being.

Figure legends

Figure 1: Gene profile comparison between CD56^{bright} and CD56^{dim} NK cells derived from HD and two TAP-deficient patients.

Experimental conditions: NK cells (CD56^{bright} and CD56^{dim}) were sorted from freshly defrosted PBMC derived from 4 HD and 2 TAP-deficient patients, before performing whole genome microarrays. Differentially expressed genes were determined using a *p*-value (FDR) < 0.05, and no Fold Change abs(FC). (**A**) Heat maps represent the differentially expressed genes between the two NK cell types (HD and TAP-deficient) and the 2 differentiation stages (CD56^{bright} and CD56^{dim}). (**B**) Scheme represents the differentially expressed gene numbers in the 5 different combinations when comparing the 2 cell types (HD and TAP-deficient), and the 2 stages (CD56^{bright} and CD56^{dim}). In parenthesis are indicated the differentially-expressed-gene clusters with an enrichment score > 2, calculated with Database for Annotation, Visualization and Integrated Discovery (DAVID) v6.7, available on http://david.abcc.ncifcrf.gov/. Blue numbers indicate the punctual comparisons (HD CD56^{bright} *vs* TAP-deficient CD56^{bright}, and HD CD56^{dim} *vs* TAP-deficient CD56^{dim}). Red numbers indicate the gene numbers when comparing the 2 NK stages in the same cell type (CD56^{bright} and CD56^{dim} in HD or TAP-deficient), and mauve numbers represent the difference when comparing [CD56^{bright} *vs* CD56^{dim} in HD] *vs* [CD56^{bright} *vs* CD56^{dim} in TAP-deficient patients].

Figure 2: Fresh TAP-deficient NK cells exhibit hyporesponsiveness toward K562 cells, but increase their activity after activation with IL-2.

In the graphs, black lines represent the cytotoxicity of HD NK cells, the blue lines represent the cytotoxicity of TAP-deficient patient's 1 NK cells, and the red lines are for TAP-deficient patient's 2 NK cells. NK cell cytotoxicity experiments were performed according to the protocol in [525] using the ratios 1:1, 5:1 and 10:1. For HD, n=6 in (**A**), and n=3 in (**B** and **C**). (**A**) Fresh TAP-deficient NK cells are hyporesponsive toward K562 cells, contrary to the ones derived from HD. (**B**) After overnight activation with 200U/mL of rhIL-2, HD NK cell cytotoxicity significantly increased, and TAP-deficient patient NK cells were able to kill K562 cells, but less than HD. (**C**) Following 5 days of activation with rhIL-2, both NK cell types were highly cytotoxic and were able to kill between 90% and 99% of K562 cells.

Figure 3: Gene profile comparison between fresh, 1h activated and 5h activated CD56^{dim} NK cells derived from HD and 2 TAP-deficient patients.

Experimental conditions: CD56^{dim} NK cells were sorted from freshly defrosted PBMC derived from 4 HD and 2 TAP-deficient patients, co-incubated with sorted live K562 cells during 1 hour or 5 hours, then sorted again before performing whole genome microarrays. Differentially expressed genes were determined using a *p*-value (FDR) < 0.05, and no Fold Change abs(FC). (**A**) Heat maps represent the differentially expressed genes between the 2 NK cell types (HD and TAP-deficient) and the 3 incubation times (0h, 1h and 5h). (**B**) Scheme represents the differentially expressed gene numbers in the 11 different combinations when comparing the 2 cell types (HD and TAP-deficient), and the 3 incubation times (0h, 1h and 5h). In parenthesis are shown the differentially-expressed-genes' clusters with an enrichment score > 2, calculated with Database for Annotation, Visualization and Integrated Discovery (DAVID) v6.7, available on http://david.abcc.ncifcrf.gov/. Blue numbers indicate the punctual comparisons (0h HD *vs* 0h TAP-deficient, 1h HD *vs* 1h TAP-deficient, and 5h HD *vs* 5h TAP-deficient). Red numbers indicate the gene numbers when comparing 2 incubation times in the same cell type (0h *vs* 1h, 1h *vs* 5h, and 0h *vs* 5h in HD, or in TAP-deficient), and mauve numbers represent the differences when comparing ([0h *vs* 1h in HD] *vs* [0h *vs* 1h in TAP-deficient patients], [1h *vs* 5h in HD] *vs* [1h *vs* 5h in TAP-deficient patients], [0h *vs* 5h in HD] *vs* [0h *vs* 5h in TAP-deficient patients]).

Figure 1

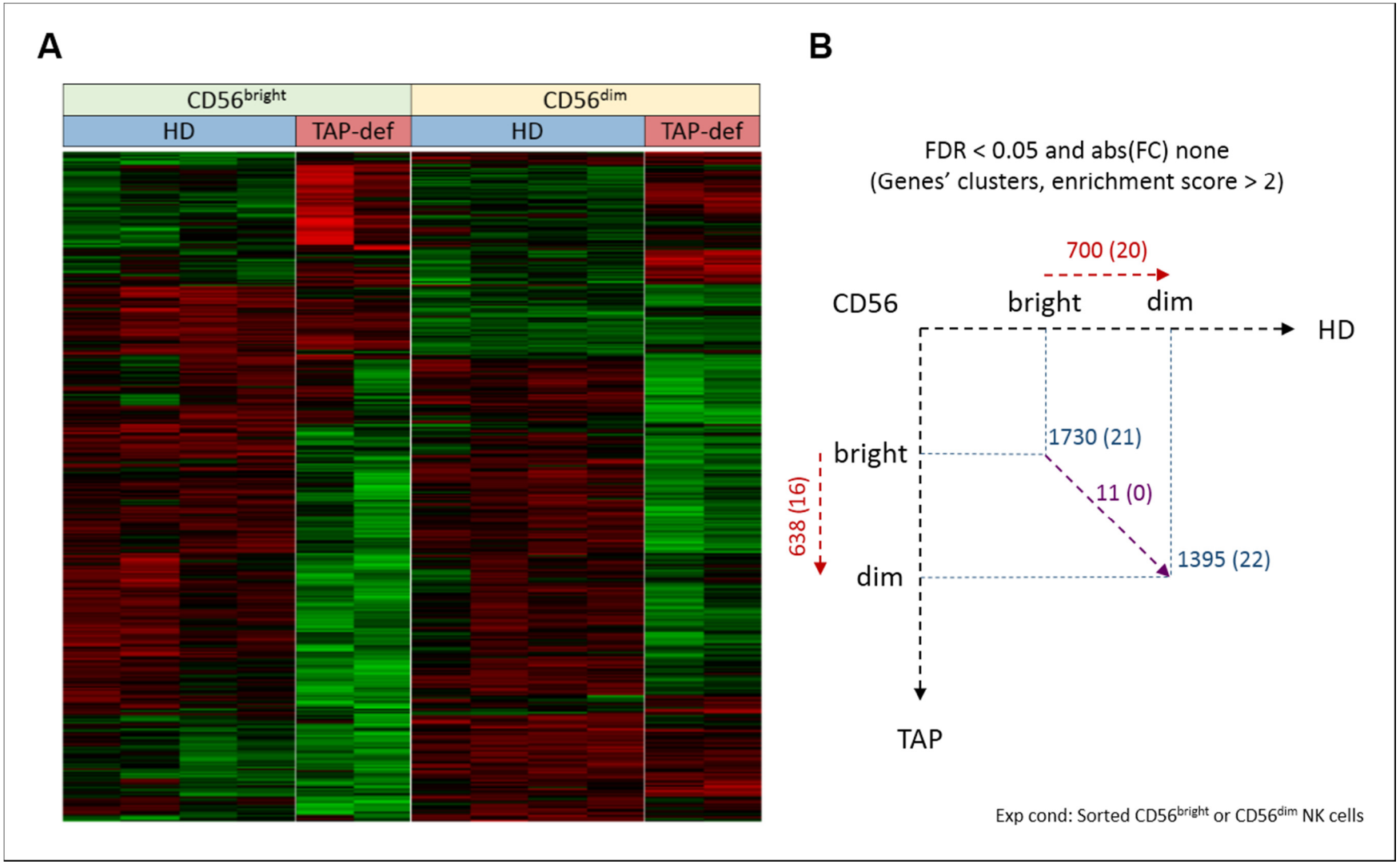

A
CD56bright
CD56dim
HD
TAP-def
HD
TAP-def
B
FDR < 0.05 and abs(FC) none
(Genes' clusters, enrichment score > 2)
700 (20)
CD56
bright
dim
HD
1730 (21)
bright
11 (0)
638 (16)
1395 (22)
dim
TAP
Exp cond: Sorted CD56bright or CD56dim NK cells

Figure 2

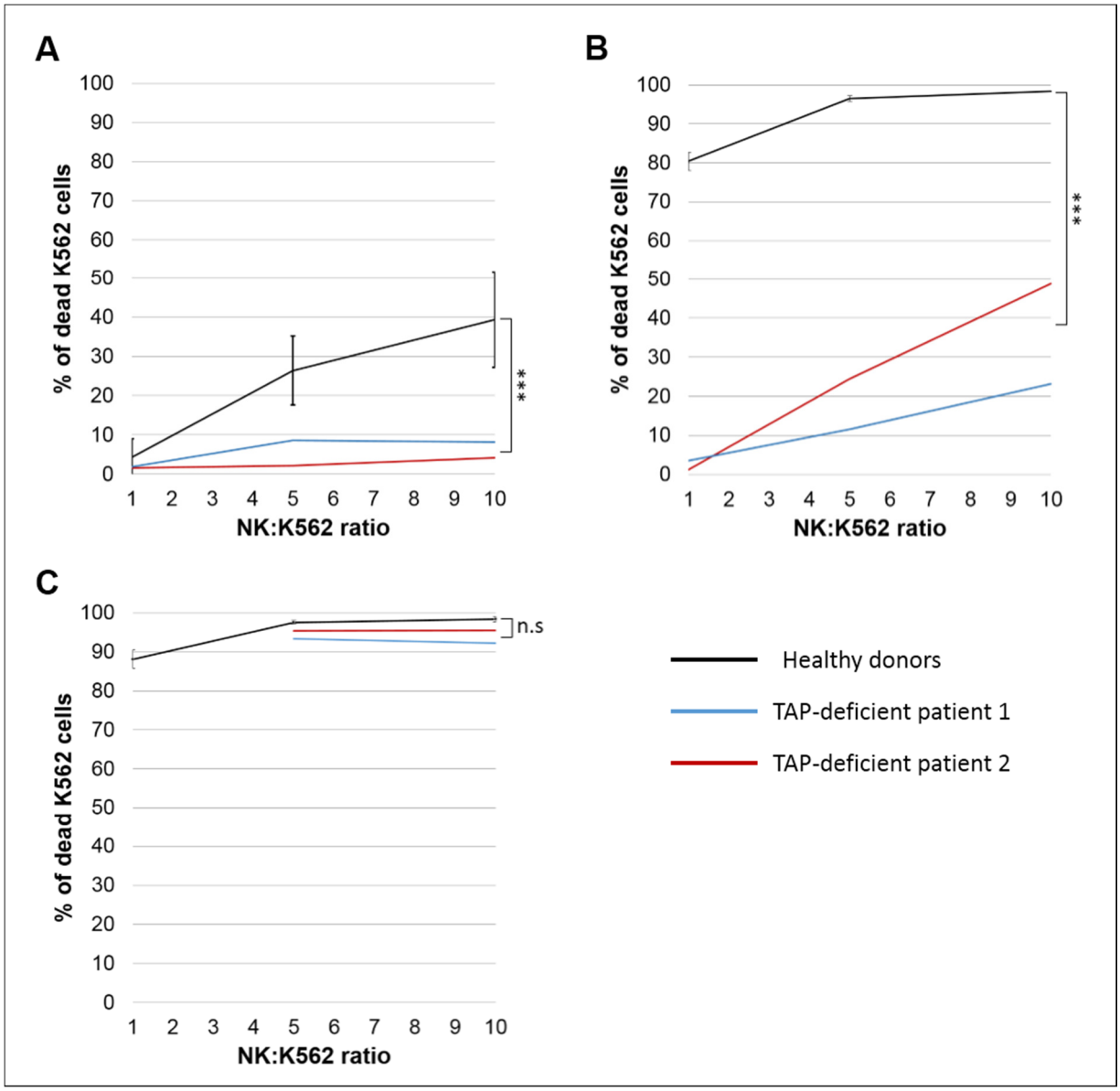
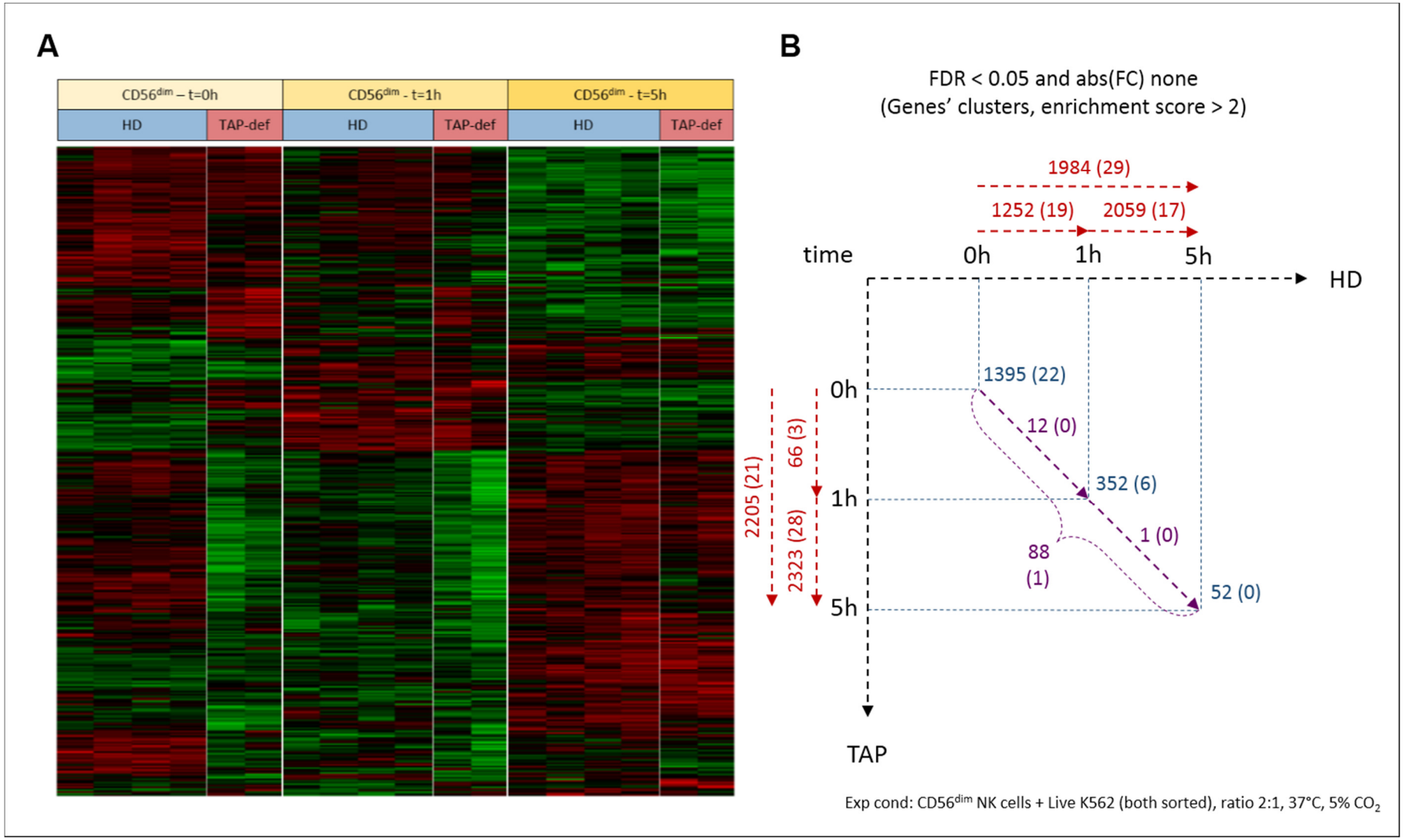

A
% of dead K562 cells
NK:K562 ratio
***
B
% of dead K562 cells
NK:K562 ratio
***
C
% of dead K562 cells
NK:K562 ratio
n.s
Healthy donors
TAP-deficient patient 1
TAP-deficient patient 2

Figure 3


A
CD56dim – t=0h
CD56dim - t=1h
CD56dim - t=5h
HD
TAP-def
HD
TAP-def
HD
TAP-def
B
FDR < 0.05 and abs(FC) none
(Genes' clusters, enrichment score > 2)
1984 (29)
1252 (19)
2059 (17)
time
0h
1h
5h
HD
0h
1h
5h
TAP
1395 (22)
12 (0)
352 (6)
1 (0)
52 (0)
88
(1)
2205 (21)
66 (3)
2323 (28)
Exp cond: CD56dim NK cells + Live K562 (both sorted), ratio 2:1, 37°C, 5% CO2

Letter 1

Clinical and Immunological Remarks about TAP Deficiency

Zimmer J, **Sleiman M**, Hentges F. and Gadola SD.
Journal of Biological Chemistry. 2012 Aug 3;287(32):27047

Comment on:

Role of Metalloproteases in Vaccinia Virus Epitope Processing for Transporter Associated with Antigen Processing (TAP)-Independent Human Leukocyte Antigen (HLA)-B7 Class I Antigen Presentation.

Lorente E, García R, Mir C, Barriga A, Lemonnier FA, Ramos M. and López D.
Journal of Biological Chemistry. 2012 Mar 23:287(13):9990-10000

Abstract

The transporter associated with antigen processing (TAP) enables the flow of viral peptides generated in the cytosol by the proteasome and other proteases to the endoplasmic reticulum, where they complex with nascent human leukocyte antigen (HLA) class I. Later, these peptide-HLA class I complexes can be recognized by CD8(+) lymphocytes. Cancerous cells and infected cells in which TAP is blocked, as well as individuals with unusable TAP complexes, are able to present peptides on HLA class I by generating them through TAP-independent processing pathways. Here, we identify a physiologically processed HLA-E ligand derived from the D8L protein in TAP-deficient vaccinia virus-infected cells. This natural high affinity HLA-E class I ligand uses alternative interactions to the anchor motifs previously described to be presented on nonclassical HLA class I molecules. This octameric peptide was also presented on HLA-Cw1 with similar binding affinity on both classical and nonclassical class I molecules. In addition, this viral peptide inhibits HLA-E-mediated cytolysis by natural killer cells. Comparison between the amino acid sequences of the presenting HLA-E and HLA-Cw1 alleles revealed a shared structural motif in both HLA class molecules, which could be related to their observed similar cross-reactivity affinities. This motif consists of several residues located on the floor of the peptide-binding site. These data expand the role of HLA-E as an antigen-presenting molecule.

LETTER

Clinical and Immunological Remarks about TAP Deficiency

We read with interest the article by Lorente *et al.* (1) describing the presentation of TAP-independent antigens from vaccinia virus by TAP-deficient cells. Their findings offer a mechanistic explanation for the fact that TAP-deficient patients are not particularly susceptible to viral infections and for why adult patients often have normal $CD8^+$ T-cell numbers (2–4). Conversely, severe viral infections might occur early in life, preparing the grounds for the usually observed recurrent bacterial infections. TAP-independent peptides might be generated during most viral infections. However, the presentation of these peptides to $CD8^+$ T-cells to generate efficient antiviral immune responses is still likely to be dependent on the patient's HLA haplotype. Interestingly, HLA-A*02, the most common HLA class I allele in Europe, has been previously shown to present diverse TAP-independent peptides to $CD8^+$ T-cells (5) but has not been found in the clinically immunodeficient patients. Thus, HLA-A*02 homozygous TAP-deficient people might be protected from severe clinical manifestations, thereby "escaping" diagnosis.

We suggest slightly modifying the statement that "TAP-deficient patients live normal life spans with only a limited susceptibility to chronic respiratory bacterial infections." Although we agree that this is possible, patients often suffer from widespread bronchiectasis and respiratory insufficiency. Many develop mutilating granulomatous skin ulcers in the mid-face and on the extremities (2–4). Importantly, treatment with immunosuppressive drugs leads to progression of bronchiectasis, respiratory failure, and complications such as brain abscesses.

A question arising is whether TAP plays a role in the presentation of bacterial antigens. Future studies might examine the efficiency of this pathway in TAP-deficient patients.

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[‡]*Laboratory of Immunogenetics and Allergology, Centre de Recherche Public de la Santé (CRP-Santé), Luxembourg, Luxembourg and* [§]*University of Southampton, Southampton, United Kingdom*

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DOI 10.1074/jbc.L112.379578

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Letter 2

TAP Deficiency is Also a Cause of Bronchiectasis

Zimmer J, **Sleiman M**, Poli A, Michel T. and Hentges F.
Thorax. 2013 May;68(5):490-1

Comment on:

Immunity and Bacterial Colonisation in Bronchiectasis.

Whitters D. and Stockley R.
Thorax. 2012 Nov:67(11):1006-13

Abstract

BACKGROUND: Non-cystic fibrosis bronchiectasis is characterised by irreversibly dilated bronchi usually associated with chronic sputum production, bacterial colonisation of the lower respiratory tract, inflammation and frequent exacerbations. Irrespective of the underlying cause, this represents failure of the host defence to maintain sterility of the respiratory tract.

OBJECTIVE: To review the interactions and associations of non-cystic fibrosis bronchiectasis with the innate and adaptive immune systems with particular emphasis on known failure of local defences established deficiencies of the adaptive immune system. In addition we wished to explore potential subtle changes in the host defence which can lead to bacterial colonisation together with bacterial factors that aid colonisation of the lower respiratory tract and impair antibiotic response. This latter concept is considered with particular reference to Pseudomonas aeruginosa, which is often found in the airway secretions of patients with non-cystic fibrosis bronchiectasis and may act as a model for other organisms.

METHODS: An extensive literature review was undertaken to provide a comprehensive review of immunity and bacterial colonisation in non-cystic fibrosis bronchiectasis, with focus on in vitro studies examining bacterial factors which may facilitate colonisation together with potential implications for management.

CONCLUSION: These themes provide a review of the current understanding of non-cystic fibrosis bronchiectasis together with areas for future research and potential therapeutic strategies.

TAP deficiency is also a cause of bronchiectasis

We read with interest the recent bronchiectasis review by Whitters and Stockley.[1]

The authors provide a comprehensive overview of the changes in the immune response that might favour bacterial colonisation of the lower airways and in a second step initiate the vicious cycle leading to chronic inflammation and recurrent infections. Their description of the innate immune system in the lung includes antibacterial peptides, neutrophils and toll-like receptors. Although this is correct, Natural Killer (NK) cells might also be mentioned, as their role in the immune response to bacteria (besides their antiviral and antitumor functions) has been repeatedly documented.[2 3] NK cells participate in innate immune defence mechanisms through (i) the release of cytotoxic granules containing, among others, the antibacterial factors granulysin, α-defensins and cathelicidin,[2] and (ii) cytokine production that stimulates both innate and adaptive immunity. In addition, they have immunoregulatory properties able to limit excessive immune responses.[3] Thus, it is highly likely that NK cells are one of the actors participating in the pathogenesis of bronchiectasis. The same may hold true for the more recently discovered (non NK) innate lymphoid cells, but more work will be necessary to establish this.

Another important point of the review is the useful list of the causes of bronchiectasis in which transporter associated with antigen processing (TAP) deficiency is however missing. TAP proteins are crucial for peptide loading on newly synthesised Human Leukocyte Antigen (HLA) class I molecules, and the consequence of TAP deficiency is a very low cell-surface expression of these HLA class I molecules.[4 5] Clinically, besides skin ulcers, the patients suffer from chronic bacterial infections of the upper and lower airways with bronchiectasis.[4 5] Although the known cases of this immune deficiency are rare (around 30 patients reported in the literature), there might be much more, as the diagnosis of idiopathic bronchiectasis might be made in most instances without having checked for TAP deficiency (by staining of patients' peripheral blood mononuclear cells with an anti-HLA class I antibody which reveals by flow cytometry a very low expression level compared to healthy control donors, and/or the absence of HLA class I molecules by serotyping).

In our opinion, it is important to detect TAP deficiency as soon as possible in order to optimise the treatment of the patients, and thus it should be systematically included in the diagnostic algorithms applied to the exploration of chronic respiratory infections and bronchiectasis.

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Contributors All the authors deserve criteria for authorship. They all read and approved the final version of the manuscript.

Funding The work of the F. Hentges lab is financed by the Luxembourgish Ministry of Health. MS is the recipient of a PhD AFR grant from the Luxembourgish National Research Fund (Fonds National de la Recherche).

Competing interests None.

Provenance and peer review Not commissioned; internally peer reviewed.

To cite Zimmer J, Sleiman M, Poli A, *et al*. *Thorax* 2013;**68**:490–491.

Received 27 November 2012
Accepted 17 December 2012
Published Online First 8 January 2013

► http://dx.doi.org/10.1136/thoraxjnl-2012-203138

Thorax 2013;**68**:490–491.
doi:10.1136/thoraxjnl-2012-203052

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COLLABORATIONS AND ONGOING PROJECTS

NK cell capacities to kill tumour and virally infected cells made them of high and continuous interest in the immunology field. During my thesis work, and taking the advantage of our developed flow cytometry facility as well as our knowledge in primary immunodeficiencies (more specifically TAP-deficiency), we had the chance to start different collaborations aiming at better understanding NK cell functions in different disease contexts.

i. Role of NKG2C in TAP-deficient individuals

Aiming at studying the role and function of NKG2C in the TAP-deficiency context, a collaboration with Prof Karl-Johan Malmberg, from the Centre of Infection Medicine (CIM) Karolinska Institutet, Stockholm, Sweden, was launched. The first set of experiments was performed by Dr Vivien Béziat Centre of Infection Medicine (CIM) Karolinska Institutet, Stockholm, Sweden) on PBMC derived from two TAP-deficient patients. This collaboration was later on extended with Oslo medical hospital where Dr Jodie Goodridge is performing experiments on TAP-deficient fibroblasts.

ii. NK cells in Multiple Myeloma (MM)

Multiple Myeloma (MM) is a haematological malignancy responsible of 2% of deaths related to cancer. Its annual incidence, which is increasing by time, is partially balanced by an improvement of the patients' survival rate especially due to immunomodulators use in treatment. Interest on the effect of these immunomodulators on the immune system function is increasing, especially in the NK cell field. Willing to better understand the NK cell/plasma cell interaction in MM, a collaboration was started with Dr Nassera Aouali (Laboratory of Experimental Hemato-Oncology, CRP-Santé Luxembourg). The project was presented for funding to the "Cancer Research Foundation in Luxembourg" and is awaiting approval.

iii. Identification of two potential new TAP-deficient patients

TAP-deficiency is a very rare disease unfamiliar to medical doctors. During my PhD studies, we tried to get in contact with many doctors around the world hoping to identify new cases allowing us to better study NK cell functions in this particular context. As a result, we started two collaborations in order to identify two potential new cases of TAP-deficiency.

iii.a - Case number 1: a woman living in Brazil and presenting the common symptoms

of TAP-deficient patients, as well as a defective surface expression of HLA class I molecules, was identified by Dr Dewton Vasconcelos. The mutation responsible of her surface-expression deficiency of HLA class I molecules has not yet been identified, but we were able to perform phenotypic and functional tests on her NK cells.

iii.b - Case number 2: till today, all the reported cases of TAP-deficiency were identified in consanguineous families which resulted in a homozygous TAP1 or TAP2 mutation. Dr Steven Holland, from the Laboratory of Clinical Infectious Diseases, NIAID, NIH, U.S.A, identified a new patient (27 years old man) with a defective surface expression of HLA class I molecules. Genetic tests showed a heterozygous mutation in TAP2 exon 4. The patient's biological material was sent to our laboratory in order to find the second mutation and perform phenotypic and functional tests on his NK cells.

iv. NK cell education by IR:MHC class I *cis* interactions

In June 2010 I started my PhD thesis at the Laboratory of Immunogenetics and Allergology (LIGA) in order to study the molecular mechanisms implicated in murine NK cell education by MHC class I molecules. Using TAP1-KO NK cells following their H2-D^d uptake form environmental cells by trogocytosis, we were planning to study the effect of the Ly49A:H2-D^d *cis* interaction on NK cell education. To do so, a collaboration was launched with Dr Petter Höglund (Center for Hematology and Regenerative Medicine, Karolinska Institutet, Stockholm, Sweden), who provided us with the EL4-D^d-GFP cell line [504]. All the preliminary experiments were successfully performed, but this project had to be postponed as the commercialisation of the major antibody used in the experiments (Ly49A-JR9-318) was stopped. In this thesis chapter we will present the experiments' results and propose a possible continuation of this project.

Role of NKG2C in TAP-Deficient Individuals

Collaboration with Prof Karl-Johan Malmberg
Centre of Infectious Medicine, Karolinska Institutet, Stockholm, Sweden

The role of HLA class I in NK cell education and functions is well established, and our most recent paper demonstrated these molecules' strong effect on KIR repertoire formation [525]. In the last few years, many studies underlined the effect of CMV infection on NK cell KIR repertoire adaptation; an expansion of the NKG2C$^+$ self-specific-KIR$^+$ NK cell population was found in CMV$^+$ patients [495, 496, 501]. In parallel to the HLA-E phenotype performed during our paper's experiments [525], NKG2C expression frequency was determined. Results showed heterogeneity in TAP-deficient NK cell profile: NKG2C was expressed on NK cells derived from three out of seven patients. As patients' CMV status in unknown, no conclusion concerning the NKG2C$^+$ NK cell population can be drawn.

In the light of these results, a collaboration was launched with Pr. Karl-Johan Malmberg (Centre of Infectious Medicine, Karolinska Institutet, Stockholm, Sweden) to whom PBMC derived from two TAP-deficient patients (presenting the highest frequency of NKG2C$^+$ NK cells) were sent, and experiments were performed by Dr Vivien Béziat. The aim of this work was to study NKG2C in TAP-deficient patients: co-expression profile of a large KIR panel on NKG2C$^+$ NK cells and role of this activating receptor in patients' NK cells.

In depth KIR phenotypic studies revealed strong differences between CMV$^+$ HD NK cells and ones derived from the two TAP-deficient patients (Fig. 1A). While NKG2C$^+$ NKG2A$^-$ (but not NKG2C$^-$ NKG2A$^+$ and NKG2C$^-$ NKG2A$^-$) NK cells showed a bias toward self-specific KIR expression in the CMV$^+$ HD (Fig. 1B), no bias in KIR expression was observed in the three former NK cell subpopulations of TAP-deficient patients (Fig. 1C, D).

To assess the functionality of NK cells expressing exclusively NKG2A or NKG2C, IFN-γ and TNF-α production, as well as degranulation (CD107a expression) toward 721.221-AEH (721.221 cell line presenting the HLA-E epitope recognised by NKG2A leading to NK cell inhibition, or NKG2C leading to NK cell activation) cells and P815+anti CD16 were performed (Fig.2). As expected, both HD NK cells (NKG2A$^+$ NKG2C$^-$ and NKG2A$^-$ NKG2C$^+$) performed rADCC following P815+anti CD16 co-culture, whereas TAP-deficient NK cells exhibited low to very low response. NKG2A$^+$ NKG2C$^-$ NK cell co-culture with 721.221-EAH cells did not result in any response for both statuses (healthy and TAP-deficient); NKG2A being an inhibitory receptor. On the other hand, NKG2A$^-$ NKG2C$^+$ HD NK cells were activated by 721.221-EAH cells. NKG2C was engaged in an interaction with its ligand leading to NK cell activation. On the contrary, both patients' NK cells (NKG2A$^-$ NKG2C$^+$) had very low response toward

721.221-AEH cells. NKG2C does not seem to act like an activating receptor in TAP-deficient individuals. This conclusion was confirmed in receptor crosslinking experiments where NK cells were incubated in plates coated with the different combinations of NKG2C, 2B4 and DNAM-1 antibodies (Fig. 3). Compared to CMV^+ HD, neither NKG2C nor the combination of 2B4 and DNAM-1 were able to activate TAP-deficient NK cells. Only combinations of NKG2C with another antibody (2B4 or DNAM-1) lead to TAP-deficient NK cell degranulation, patient 1 NK cells being more functional than the ones derived from patient 2. These results clearly demonstrate that NKG2C does not act as an activating receptor in TAP-deficient NK cells but more like an activating co-receptor.

In HD, engagement of NK cell AR, including NKG2C, induces a signal transduction through their adaptor molecule DAP12 leading to a series of protein phosphorylation by SYK and ZAP-70 resulting in Ca^{2+} signalling and NK cell degranulation. On the contrary, 2B4, DNAM-1 and NKG2D engagement with their ligand is not sufficient for activating NK cells, two signals need to synergise in order to efficiently phosphorylate the lymphocyte cytosolic protein 2 (SLP-76) thus leading to an activating signal pathway. Investigating the expression and the phosphorylation of the different molecules implicated in these pathways should allow us to better elucidate this functional difference.

In order to give more insight to the role of CMV infection on HLA class I (HLA-A, -B and -C) and HLA-E expression, a collaboration was launched with Oslo Medical Hospital, where Dr Jodie Goodridge is working on infecting TAP-deficient fibroblasts with CMV.

Figure legends

Figure 1: KIR co-expression frequency on NKG2C+ NKG2A$^-$ TAP-deficient NK cells.

(**A**) Shown is the frequency of NKG2C$^+$ NKG2A$^-$ CD56^{dim} NK cells in seven TAP-deficient patients [525] and a panel of healthy donors. Only three patients (1, 2 and 5) exhibited high levels of NKG2C$^+$ NKG2A$^-$ CD56^{dim} NK cells. The expression frequency of seven different KIR molecules was determined on NKG2C$^-$ NKG2A$^-$, NKG2C$^-$ NKG2A$^+$, and NKG2C$^+$ NKG2A$^-$ CD56^{dim} NK cells in one healthy donor (**B**) and TAP-deficient patients 1 and 2 (**C** and **D** respectively). The presence of one KIR in a combination is represented by a colour code below the graph: 2DL1 (dark blue), 2DL2/S2 (purple), 2DL3 (red), 2DS1 (light blue), 2DS4 (orange), 3DL1 (green), 3DL2 (black). Data in (**A**) were analysed with Kaluza analysis software v1.2 (Beckman Coulter), and in (**B**) with the Boolean gate algorithm of Flow Jo Version 9.6 (TreeStar). Pestle software Version 1.6 was used to remove the background.

Figure 2: Functional characterization of NKG2C+ NKG2A$^-$ NK cells in TAP-deficient patients.

Polyfunctional assays were performed on thawed PBMC, rested overnight and stimulated by HLA-E expressing 721.221.AEH cells or P815 coated with 1µg/mL anti-CD16 (3G8 clone). Cells were stained with mAbs for CD107a (degranulation), and intracellular expression of IFN-γ and TNF-α. Pie charts show the frequency of NK cells positive for 0, 1, 2, or 3 responses (CD107a, IFN-γ, and TNF-α). The color-coded arcs depict the frequency of cells positive for CD107a, IFN-γ, and TNF-α. Data were analysed with the Boolean gate algorithm of Flow Jo Version 9.6 (TreeStar). Pie charts were generated using the Spice software Version 5.3 (NIAI Freeware).

Figure 3: NKG2C is an activating co-receptor in TAP-deficient patients.

Degranulation assays were performed on thawed PBMC, rested overnight and stimulated by coated antibodies corresponding to the eight possible combinations of NKG2C, DNAM-1 and 2B4. White histograms represent a panel of healthy donors, grey histograms represent TAP-deficient patient 1, and black histograms represent TAP-deficient patient 2. Data were analysed with the Boolean gate algorithm of Flow Jo Version 9.6 (TreeStar). Histograms represent the mean ± SD.

Figure 1:

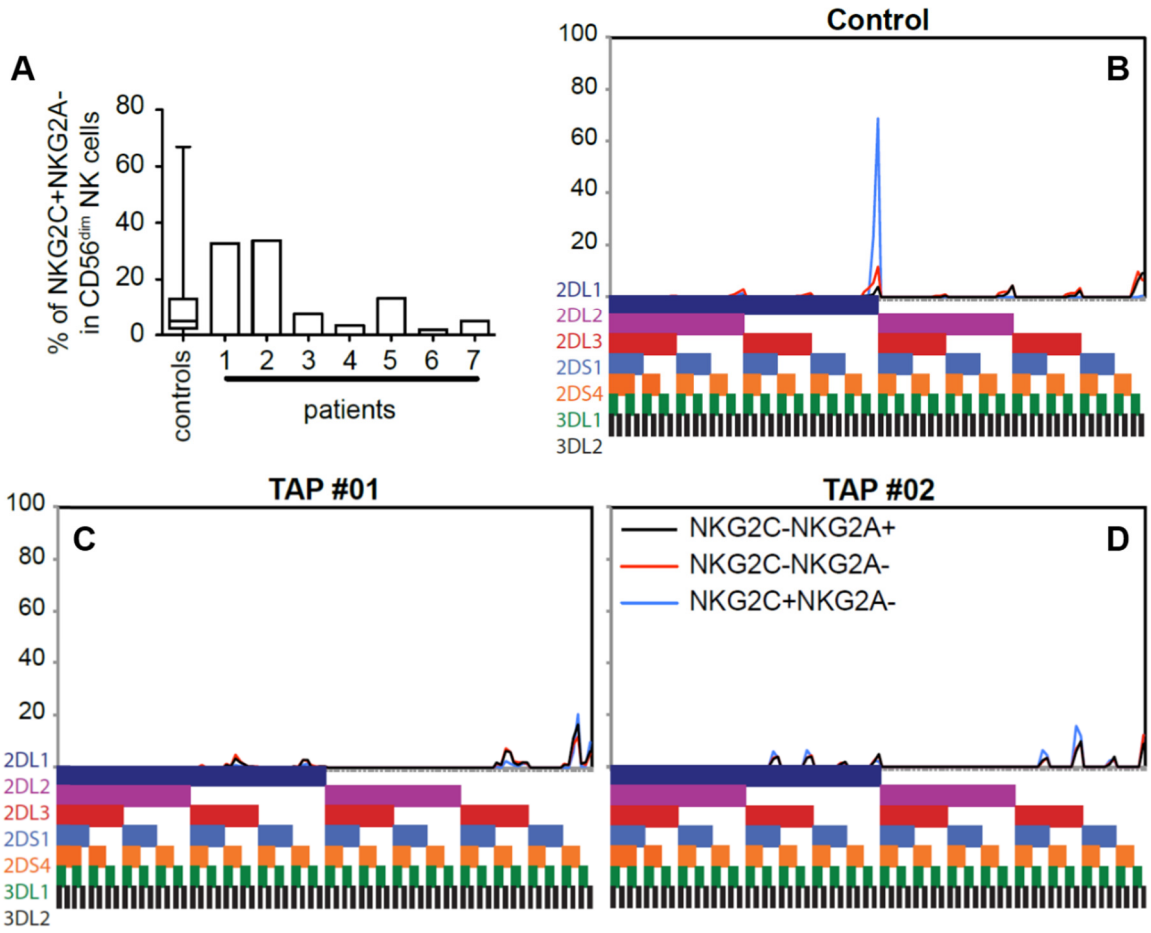

A
% of NKG2C+NKG2A- in CD56dim NK cells
80
60
40
20
0
controls
1 2 3 4 5 6 7
patients
Control
B
100
80
60
40
20
2DL1
2DL2
2DL3
2DS1
2DS4
3DL1
3DL2
TAP #01
C
TAP #02
D
NKG2C-NKG2A+
NKG2C-NKG2A-
NKG2C+NKG2A-

Figure 2:

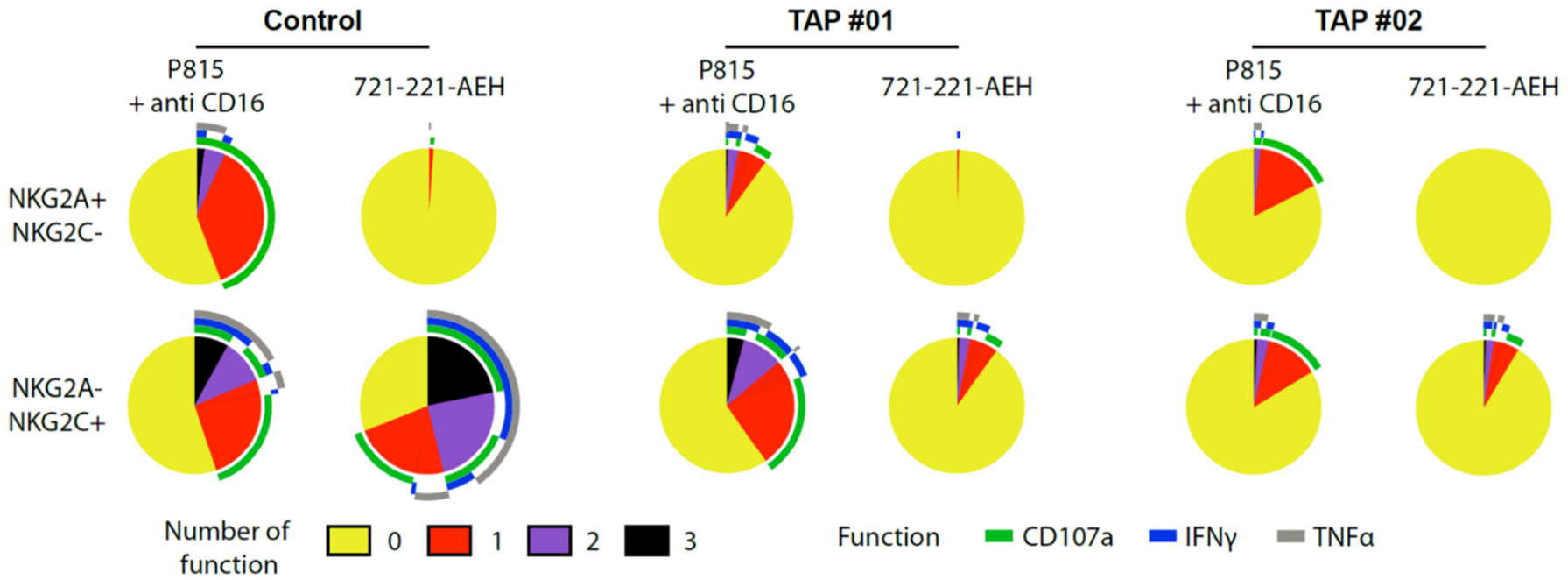
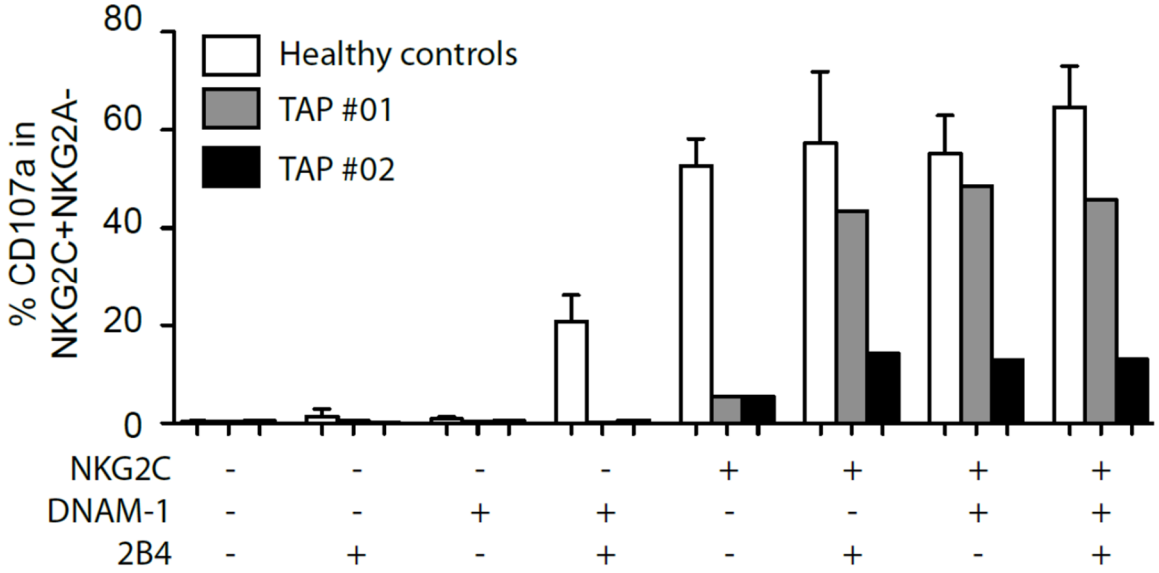

Control
TAP #01
TAP #02
P815
+ anti CD16
721-221-AEH
NKG2A+
NKG2C-
NKG2A-
NKG2C+
Number of
function
0
1
2
3
Function
CD107a
IFNγ
TNFα

Figure 3:


80
60
40
20
0
% CD107a in NKG2C+NKG2A-
Healthy controls
TAP #01
TAP #02
NKG2C - - - - + + + +
DNAM-1 - - + + - - + +
2B4 - + - + - + - +

NK Cells/plasma cells interactions in Multiple Myeloma (MM)

Collaboration with Dr Nassera Aouali
Laboratory of Experimental Hemato-Oncology, CRP-Santé Luxembourg

Multiple Myeloma (MM) is a type of haematological cancer distinguished by high proliferation levels of clonal plasma cells in the BM. Plasma cells are differentiated B lymphocytes responsible of Ig production that play a major role in humoral adaptive immune response [527]. Hence, due to malignant plasma cell accumulation, the smooth production of platelets, erythrocytes and leukocytes is disturbed. Ig production is also affected and overproduction of a non-functional protein (Ig) takes place, also known as paraprotein or monoclonal protein (M protein or M Ig) [528].

MM diagnosis is based on different criteria which includes symptoms of bone disease, renal failure, anaemia, bleeding, recurrent bacterial infections, and laboratory tests such as plasma hyperviscosity, raised erythrocyte sedimentation rate and over production of M serum protein or globulin. When MM is suspected, further verification of the disease should be carried out by BM aspiration plasma cells count [529] and infiltration assessment by immunohistochemistry on a trephine section biopsy of 20mm length [530]. Normal levels of plasma cells in HD BM is around 2%, and can reach to 10% in early stages of the disease. MM evolution can be divided into four different stages: two asymptomatic stages which could evolve to multiple myeloma (MM), monoclonal gammopathy of undetermined significance (MGUS) and smoldering multiple myeloma (SMM), MM could be progress in plasma cell leukaemia (PCL). In the last stages of the disease, plasma cells pass from the BM to the blood and constitute more than 20% of blood lymphocytes. The median age of individuals presenting MM is approximately 70 years. The annual incidence of newly diagnosed cases of MM is increasing, but the survival rate is improving by time [531] especially thanks to a new class of drugs used in MM treatment, the immunomodulators.

Immunomodulators, currently used in MM treatment, include “Lenalidomide”. These drugs lead to an increase or reduction of the immune system response, in this case towards plasma cells. The immunomodulators’ implications on the immune system, more specifically in the MM context, is growing by time [532-535]. The aim of this study is to better understand the mechanisms behind MM resistance to NK cell killing throughout the disease evolution, and study the effect of the different immunomodulators on NK cell functions. To do so, blood and BM sampling will be carried out on patients from the different stages of the disease; MGUS, SMM, MM and PCL, followed by deep NK cell phenotype and functional tests. Immunomodulators’ combinations cytotoxicity will be also studied on both plasma cells and

NK cells. Results should give us more information about the interactions between plasma cells and NK cells in the MM context and help us propose more adapted clinical treatments.

Identification of Two New TAP-deficient Patient

During my PhD thesis work, we were able to get in contact with two doctors who identified patients presenting defective surface expression of HLA class I molecules. Our main part of the collaboration was to identify the mutation and perform different NK cell studies. In this chapter, we will present two different case studies on which work is still ongoing.

Case study I:

Collaboration with Dr Dewton Vasconcelos,
Laboratory of Medical Investigation, University of São Paulo School of Medicine, Brazil

TAP-deficient cases are very rare: 30 cases were described till today. This study aims at investigating a new patient presenting a HLA class I surface expression defect by:

(i) sequencing the genomic DNA region of TAP-1 and TAP-2, hoping to find a mutation explaining the patient's phenotypic status.

(ii) performing a detailed phenotypic and functional study on NK cells (based on the experiments in Sleiman *et al.* 2014 [525]) in order to compare this new patient's results to the former ones.

In this case report we describe a potential new TAP-deficient patient, from Brazil, with a confirmed defective expression of HLA class I molecules (94% reduction of HLA class Ia, and 75% reduction for HLA-E) (Fig. 1A). She represents the common symptoms of TAP deficiency: granulomatous skin ulcers and recurrent upper airways' infections. In collaboration with Dr Carlos Vilches (University Hospital Puerta de Hierro, Madrid, Spain) KIR and HLA class I genotyping were performed, showing a homozygous HLA profile correlating with her parents' consanguineous marriage (Table 1A, B). Primers situated in the 5' and 3' intronic sequences of TAP-1 and TAP-2 exons were designed, and their sequencing is ongoing in collaboration with Prof Claude Müller and Dr Jonathan Turner (CRP-Santé, Department of Immunology, Luxembourg).

To assess the HLA class I defective expression impact on their receptors' expression and co-expression, in-depth phenotypic studies were performed as described in [525] (Fig. 1B, C and D). Surprisingly, no differences were observed in CD8, NKG2A, CD57 and KIR co-expression frequency between patient Case I and HD (Fig. 1B). A co-expression frequency study of CD8/NKG2A/KIR (Fig. 2C) and CD57/NKG2A/KIR (Fig. 2D) confirmed these

similarities. Patient Case I presents the same expression frequencies as HD, and constitutes the second outlier in our 8 patients with HLA class I defective surface expression. Only an over representation of NKG2A^{-} 0 KIR NK cells was observed in the CD57^{+} NK subpopulation.

Regardless of the patient's mutation (which is still not identified), her NK cells were found to be hyporesponsive toward K562 cells: low cytotoxicity (Fig. 2A), and no IFN-γ production nor NK cell degranulation were observed (data not shown). The presence of cytotoxic molecules (granzyme B, perforin and granulysin) was analysed, and their expression frequency in CD56^{dim} NK cells was similar to both HD and the former 6 TAP-deficient patients described in our previous paper [525].

In this study, we describe a new patient presenting the typical clinical symptoms of TAP-deficiency. Her NK cells exhibit hyporesponsiveness towards K562 cells, but no major differences in their HLA class I receptor expression frequency compared to HD were observed. While working on identifying the mutation causing her HLA class I phenotype, further studies on her NK cells could give us more information on her repertoire formation and NK cell functions.

Case study II:

Collaboration with Dr Steven Holland,
Laboratory of Clinical Infectious Diseases, NIAID, NIH, USA

Till today, the described cases of TAP-deficiency, resulting from consanguineous parents, were all homozygous for the TAP-1 or TAP-2 mutations as well as for their HLA genotypes. Two years ago, Dr Steven Holland (Laboratory of Clinical Infectious Diseases, NIAID, NIH, USA) identified a new patient (29 years old male) presenting a chronic progressive granulomatous dermatitis on his upper left arm. This patient is of Northern European descent born to non-consanguineous parents, and is CMV negative. Different therapies were used in treating his skin granulomatous dermatitis, like complete excision and grafting of the affected skin, as well as treatment with systemic and topical corticosteroids, without any success. PBMC staining showed a defective expression of HLA class I molecules (67% reduction compared to HD). Following the sequencing of TAP-1 and TAP-2 mRNA, a mutation was found in exon 4 of TAP-2 resulting in a premature STOP codon (c.817C>T). Another mutation, in the exon 8 region of TAP-2 is suspected but still not identified.

Our laboratory was contacted in order to help finding the second mutation and perform

NK cell studies. Due to a transport technical problem, PBMC were received in a bad condition, so NK cell studies were postponed to the next annual patient's blood sampling. The patient's KIR and HLA genotyping, in collaboration with Dr Carlos Vilches (University Hospital Puerta de Hierro, Madrid, Spain) confirmed his HLA heterozygous profile (Table 1). Studies are still ongoing, hoping to identify the second TAP-2 mutation.

Figure legends

Table 1: KIR and HLA class I and class II genotype of the case I and case II patients

Figure 1: Case I patient expresses low levels of HLA class I and a HLA class I receptor expression pattern different form HD and other TAP-deficient patients.

(**A**) Shown is the HLA-A, -B and -C expression on CD56dim NK cells derived from one HD and the Case I patient. The same expression was observed on all PBMC. (**B**, **C** and **D**) box plots represent HD and TAP-deficient patients (reused from [525]) and red dots the patient Case I. When comparing the different markers expression and co-expression in **A**, **B** and **C**, patient Case I presents the same expression frequencies as HD except for NK cells that are $NKG2A^{-}$ 0KIR in the $CD57^{+}$ subpopulations. In figures, ****p<1x10-6, ***p<0 .001, **p<0 .01, *p<0 .05 and #p<0 .1.

Figure 2: Patient Case I exhibits hyporesponsive NK cells although they express cytotoxic molecules.

In (**A**) the black line represents HD and the red dashed line patient Case I. In (**B**) box plots represent HD and TAP-deficient patients (reused from [525]) and red dots the patient Case I. (**A**) cytotoxicity assays were performed on purified $CD56^{dim}$ NK cells and K562 cells co-incubated for 5 hours. Hyporesponsiveness, compared to HD, is observed for patient Case I. (**B**) The percentage of $CD56^{dim}$ NK cells expressing granulysin, granzyme B and perforin was determined. Patient Case I does not show major difference compared to HD NK cells. In figures, **p<0 .01, *p<0 .05 and #p<0 .1.

Table 1:

A

	HLA genotyping					
Codes	HLA I			HLA II		
Case I	A*01:01	B*08:01	C*07:01	DRB1*03:01	DQB1*02:01	DQA1*05:01
Case II	A*02 A*29	B*44:02 B*44:03	C*03 C*16	DRB1*04 DRB1*12		

B

	KIR genotype																
Codes	2DL1	2DL2	2DL3	2DL4	2DL5	3DL1	3DL2	3DL3	2DS1	2DS2	2DS3	2DS4	2DS5	3DS1	2DP1	3DP1	3DX1
Case I	pos	-	pos	pos	-	pos	pos	pos	-	-	-	pos (full lenght)	-	-	pos	pos (exon 2 del)	pos
Case II	pos	pos	pos	pos	pos	pos	pos	pos	-	pos	pos	pos (full lenght)	-	-	pos	pos (exon 2 del)	pos

Figure 1:

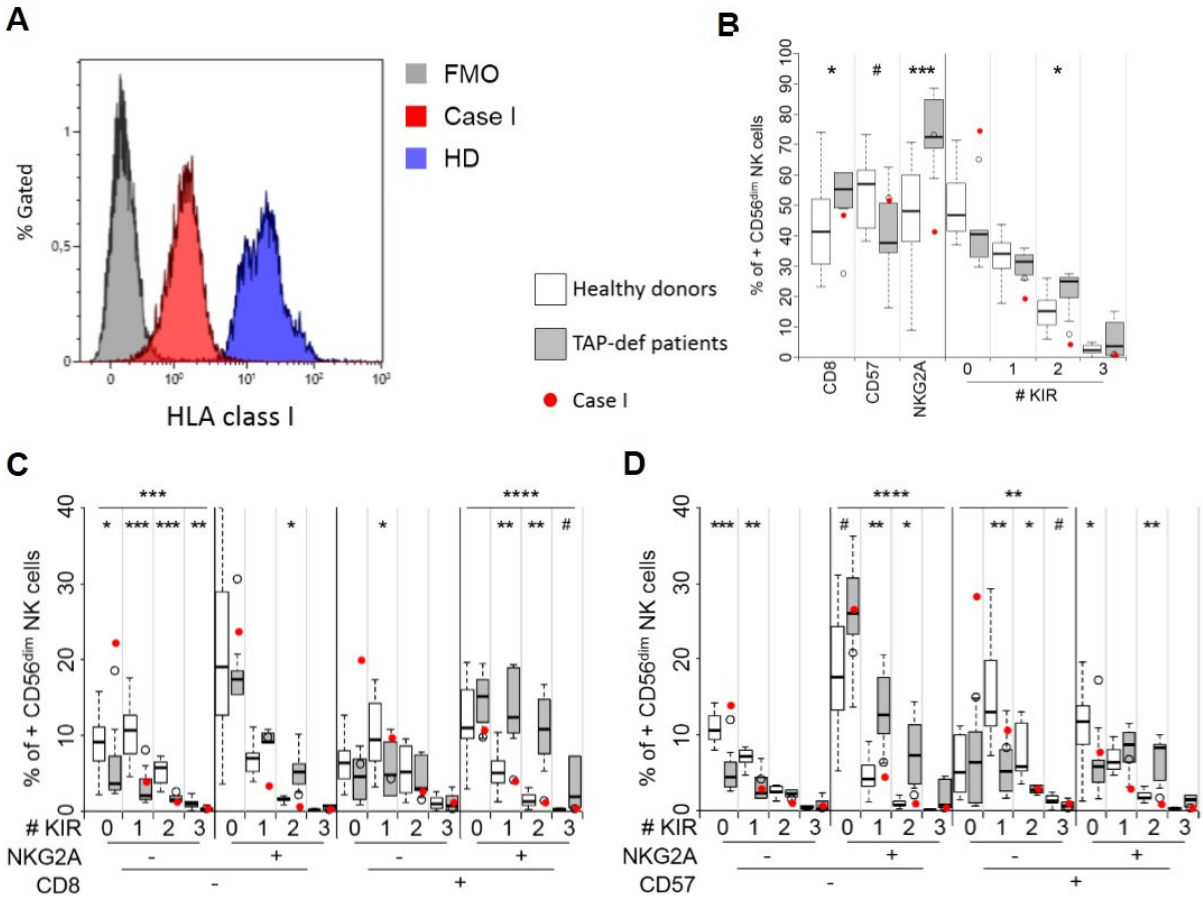
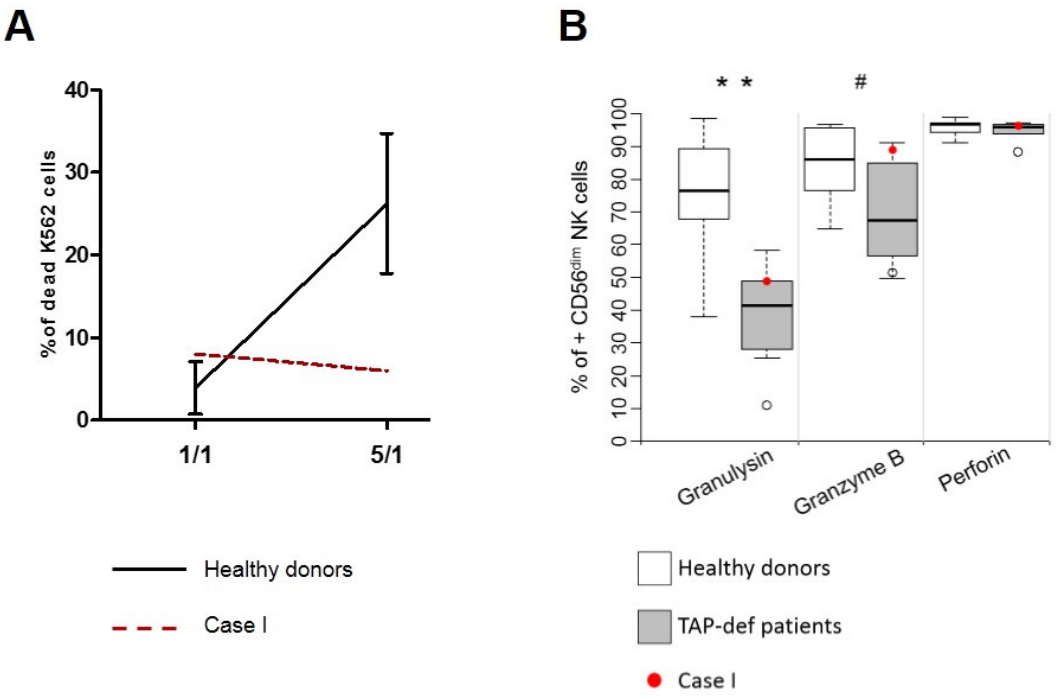


Figure 2:


A
%of dead K562 cells
40
30
20
10
0
1/1
5/1
Healthy donors
Case I
B
* *
#
% of + CD56dim NK cells
0 10 20 30 40 50 60 70 80 90 100
Granulysin
Granzyme B
Perforin
Healthy donors
TAP-def patients
Case I

NK cell education by IR:MHC class I *cis* interactions

The study of IR:MHC class I *cis* interaction on NK cell education was the main aim of this project. In order to give more insight to the mechanism behind these interactions, we:

(i) characterised phenotypically and functionally C57BL/6 and TAP1-KO NK cells

(ii) investigated the membrane transfer (trogocytosis) in both murine NK cell types

(iii) planned to compare NK cell functionality in both NK cell statuses (C57BL/6 and TAP1-KO) before and after trogocytosis.

Preface

NK cell education is an adaptive process resulting from MHC class I molecule interaction with their specific NK cell IR. Educated, thus functional NK cells have to express at least one IR specific for cognate MHC class I molecules. The interaction between the two former molecules can occur in: (i) *trans*, when each molecule is on a different cell surface, or (ii) *cis,* when both molecules are expressed on the same cell surface. Different models have been developed trying to give more insight to the NK cell education/tolerance mechanism (licensing, disarming, or rheostat models), neglecting the IR:MHC class I *cis* interactions.

In 2001, three independent studies demonstrated for the first time the existence of a membrane transfer mechanism in NK cells, named trogocytosis [440, 504, 505]. In this phenomenon, following a specific interaction of a NK cell receptor with its corresponding ligand expressed on surrounding cells, the NK cell is able to acquire this ligand and express it on its cell surface (*cf. chapter 3.7. Trogocytosis*). The result of this uptake is a NK cell surface *cis* interaction between the receptor and its newly acquired ligand. Here we aimed at studying the influence of IR:MHC class I *cis* interaction on NK cell education by performing Ly49A:H2-D^d uptake. The different experiments are described in the chapter *Materials and Methods*.

This project was the main subject of my PhD project on which I worked during the first 18 months of my thesis. The key antibody of this study is Ly49A (JR9-318) which allows us to identify the trogocytosed Ly49A$^+$ NK cells. Due to the stopping of its production by "Becton Dickinson", trogocytosis studies had to be postponed. In this chapter we will present the results we were able to obtain, and propose possible experiments which could help to complete them.

Results

C57BL/6 and TAP1-KO NK cells show differences in some of their IR expression, but not in their maturation.

NK cells derived from C57BL/6 (WT) and TAP1-KO splenocytes were selected among the live/CD3$^-$ NK1.1$^+$ cells, then the expression frequency of the different receptors was determined (Fig. 1A). Differences were only observed for KLRG1 which was significantly less expressed in TAP1-KO NK cells, and Ly49F, Ly49C/I and NKG2A/C/E which were overexpressed in the same NK cell type. NK cell maturation statuses were assessed by measuring the CD27/CD11b expression pattern (Fig. 1B). Both NK cell types presented the same subsets' distribution according to the four stages of maturation: CD11b$^-$ CD27$^-$, CD11b$^-$ CD27$^+$, CD11b$^+$ CD27$^+$, and CD11b$^+$ CD27$^-$, indicating no maturation disturbance in TAP1-KO NK cells compared to WT ones.

TAP1-KO NK cells are hyporesponsive compared to WT NK cells, although they express granzyme A.

When analysing granzyme A expression, no differences were observed by comparing both NK cell types (WT and TAP1-KO), or their education status (Fig. 2A). Edu$^+$ NK cells being Ly49C/I$^+$ NKGE2A/C/E$^+$ and Edu$^-$ NK cells are Ly49C/I$^-$ NKG2A/C/E$^-$. Both NK cell types, independent of their education status, express granzyme A, so they have part of the necessary machinery to kill target cells. Following activation with coated NK1.1 antibody, WT NK cells degranulated significantly higher than TAP1-KO ones (Fig. 2B). No response was observed for CD137 (negative control) nor IgG2a (isotype control), indicating that the response is specific for NK1.1 stimulation. Similar results were observed when TAP1-KO and WT negatively selected NK cells were co-cultured during 6h with YAC-1 target cells, ratio 2:1 with 35U/mL of rhIL-2 (Fig. 2C, D). TAP1-KO NK cells showed complete hyporesponsiveness: neither IFN-γ production nor degranulation (CD107a expression), contrary to WT NK cells which produced IFN-γ and degranulated when in contact with tumour cells. In the latter NK cell type, educated ones were more responsive than the non-educated ones.

TAP1-KO and WT NK cells take up H2-D^d from donor cells following Ly49A:H2-Dd interaction.

It was previously shown that Ly49A$^+$ NK cells are able to take up H2-D^d from surrounding cells and express it on their surface. Using the EL4-D^d-GFP cell line (EL4 cell line transfected with a plasmid expressing H2-D^d coupled to GFP [504]), we investigated the ligand uptake by NK cells. Both NK cell types expressed Ly49A (JR9-318) (18% ± 2% expression frequency) but no H2-D^d was detected (Fig. 3A1, A2). Following 1 hour of incubation with EL4-D^d-GFP cells (donor cells) at a ratio of 1/1 (NWNA spleen cells/donor cells), 97%-99% of Ly49A$^+$ NK cells acquired the ligand and became positive for H2-D^d (Fig. 3 B1, B2). In order to investigate the specificity of the interaction, donor cells were incubated with anti-H2-D^d antibody prior to the trogocytosis in order to block the ligand interaction site. Following co-incubation of NK cells with these donor cells, no double Ly49A H2-D^d double positive cells were found (Fig. 3 C1, C2). The H2-D^d transfer from donor cells to Ly49A$^+$ NK cells was inhibited as the interaction site is blocked, demonstrating the specificity of the ligand uptake. Ly49A molecules present different epitopes recognised by three different antibodies: JR9-318, YE1-48 and A1. In order to investigate which epitope interacts with H2-D^d and is responsible for the ligand acquisition, trogocytosis assay was performed followed by Ly49A staining with each of the three different Ab. The JR9-318 epitope (Fig. 3 B1, B2) was able to detect all Ly49A$^+$ NK cells, same as YE1-48 (data not shown). On the contrary, following trogocytosis, no Ly49A$^+$ (A1) NK cells were found, whereas 18% ± 2% of NK cells were H2-D^{d+}, corresponding to the Ly49A (JR9-318)$^+$ NK cell percentage (Fig. 3 D1, D2). These results clearly indicate that Ly49A1 epitope was masked following trogocytosis and could not be recognised by its Ab. H2-D^d uptake by Ly49A$^+$ NK cells is the result of the specific ligand interaction with the Ly49A1 epitope.

Discussion and perspectives

Protein exchange between lymphocytes was first described in 1973 by Bona et al. [502]. Later studies demonstrated an uptake of MHC class I and class II molecules by CD8$^+$ and CD4$^+$ T cells respectively, affecting their functions and immune response [507-509]. Such ligand uptakes were also reported in NK cells. Murine H-2D^{d-} Ly49A$^+$ NK cells were able to take up H2-D^d from surrounding cells after transfer to Balb/c mice [504], or in a transgenic mouse model expressing different levels of H2-D^d [440]. In this study, we aimed at examining H2-D^d uptake in TAP1-KO NK cells and study the effect of the resulting *cis* interaction between the receptor and the ligand on NK cell functions.

A phenotypic characterisation of both WT and TAP1-KO NK cells showed significant differences only in a few surface receptors. As previously reported [536, 537], Ly49C/I (the specific receptor of H2-K^b, the cognate MHC class I molecules of C57BL/6 mice), presented an over-expression in TAP1-KO spleen NK cells. Similar results were also found for NKG2A/C/E, specific receptors of Qa-1, a non-classical MHC class I molecule. KLRG1 was associated with a mature NK cell phenotype and a reduction of IL-15 induced-proliferation [538]. Its significantly reduced expression on TAP1-KO NK cells compared to WT ones demonstrates a lack in the former cells' maturity, which was not confirmed by the CD27/CD11b expression pattern; both NK cell statuses presented the same distribution of the four CD27/CD11b populations. In TAP-deficient NK cells, CD27/CD11b expression evolution can take place as in WT ones, but NK cell maturation is not completed due to the low expression of MHC class I molecules. These results go in the same line as our finding on human NK cells which are able to progress from $CD56^{bright}$ to $CD56^{dim}$, but the expression of CD57, a maturity marker, is reduced [525].

Functionally, TAP1-KO NK cells showed hyporesponsiveness following Ab activation and YAC-1 co-culture. On the contrary, WT NK cells produced IFN-γ and/or degranulated in both activation cases, and educated (Ly49C/I^+ NKG2A/C/E^+) NK cells were more functional than non-educated ones (Ly49C/I^- NKG2A/C/E^-) corroborating the hypothesis that NK cells need to express at least one IR specific for cognate MHC class I molecules in order to become functional [421].

Ligand uptake from surrounding cells was previously described in NK cells derived from C57BL/6, β_2m-KO and MHC class I transgenic mice [440, 504]. Trogocytosis following TAP1-KO NK cell co-incubation with donor cells were similar to the former results. Ligand uptake was independent of whether or not the NK cell expressed MHC class I molecules; both WT and TAP1-KO Ly49A^+ NK cells were able to acquire H2-D^d from donor cells. This uptake proved to be a molecular mechanism independent of temperature or the cell medium in which NK cells and donor cells are co-cultured. Following a 30 min, 1h, 2h or 4h of co-incubation in complete DMEM or PBS, at 4°C, room temperature or 37°C 5% CO_2, 97%-99% of Ly49A^+ NK cells acquired H2-D^d (data not shown). Trogocytosis specificity was demonstrated by inhibiting the uptake after H2-D^d block in donor cells by an anti-H2-D^d Ab. Ly49A1 masking following trogocytosis clearly demonstrates that H2-D^d uptake by NK cells is a result of the specific interaction of the ligand with the A1 epitope of the Ly49A receptor.

Sjöström et al. have demonstrated that RNK.Ly49A NK cells (a rat NK cell line stably transfected with the Ly49A) showed a reduced killing of YB2/0 target cells (a rat myeloma cell line) following the H2-D^d uptake. Killing was abolished when target cells expressed H2-D^d

molecules, implicating that even after H2-D^d uptake, Ly49A$^+$ NK cells can still receive inhibitory signals through *trans* interactions [504]. These results cannot be extrapolated to TAP1-KO NK cells as RNK.Ly49A NK cells are initially functional, contrary to our TAP-deficient murine NK cells. H2-D^d acquisition following trogocytosis may have different consequences when taking the recipient cell status in consideration.

The next step in our study was to investigate the functional consequences of the newly acquired Ly49A:H2-D^d *cis* interaction on murine TAP1-KO NK cells following trogocytosis. Separating both cell lines after co-incubation was necessary in order to eliminate the possible *trans* interactions between the ligand and its receptor which may interfere in the experiment's outcome. Different methods were tested, but none was a success. After co-incubation, NK cells not only acquire H2-D^d from donor cells, but also membrane patches which included CD3 (EL4 cells are CD3$^+$), so following negative selection, H2-Dd$^+$ NK cells were retained in the column alongside donor cells. FACS sorting did not solve the problem either, as sorted NK cells were very fragile and died shortly after, not giving us the possibility to test trogocytosis effect on their functions. Adding to this the production stop by "BD Biosciences" of the Ly49A (JR9-318) Ab, the continuation of our project was postponed.

Figure legends

Figure 1: Phenotypical characterisation of C57BL/6 and TAP1-KO NK cells.

Histograms represent the expression percentage mean with SD of each of the studied markers on NK cells, n=6 (**A**) Black histograms represent C57BL/6 mice, and light grey histograms represent TAP1-KO mice. NK cells (NK1.1$^+$ CD3$^-$) derived from C57BL/6 and TAP1-KO mice spleens were phenotyped for different markers including inhibitory and activating receptors. (**B**) NK cell maturation was also studied using CD27 and CD11b (MAC-1). ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$.

Figure 2: Functional characterisation of C57BL/6 and TAP1-KO NK cells following activation with NK1.1 Ab or YAC-1 cells.

Histograms represent the expression-percentage mean of each marker on NK cells. n=2 in **A**, **C** and **D**, and n=3 in **B**. In (**A**) and (**B**), black histograms represent WT NK cells and light grey histograms represent TAP1-KO NK cells. In (**C**) and (**D**), grey histograms represent all NK cells, grey histograms with diagonal lines represent educated (Edu$^+$) NK cells (Ly49C/I$^+$ NKG2A/C/E$^+$), and grey histograms with horizontal lines represent non-educated (Edu$^-$) NK

cells (Ly49C/I$^-$ NKG2A/C/E$^-$). (**A**) Shown is the granzyme A expression frequency on C57BL/6 and TAP1-KO NK cells. (**B**) Histograms represent NK cell degranulation following antibody crosslinking activation. NK1.1 is the Ab of interest, CD137 the negative control, and IgG2a the isotype control. (**C**, **D**) Shown is the NK cells' IFN-γ production and CD107a expression following co-incubation with YAC-1 cells, ratio 2:1 (NK:YAC-1), 35U/mL of rhIL-2, 6h at 37°C, 5% CO_2. $^{**}p < 0.01$.

Figure 3: TAP1-KO and C57BL/6 NK cells can specifically take up H2-D^d from the surrounding cells.

Shown are eight dot plots representing the co-expression status of Ly49A (JR9-318 clone, columns **A**, **B** and **C**, or A1 clone, column **D**) and H2-D^d on C57BL/6 (first line, **A1**, **B1**, **C1** and **D1**) and TAP1-KO (second line, **A2**, **B2**, **C2** and **D2**) NK cells. (**A1**, **A2**) NK cells were stained for Ly49A (JR9-318) before trogocytosis, (**B1**, **B2**) after trogocytosis, (**C1**, **C2**) or after trogocytosis following H2-D^d block in donor cells by an anti-H2-D^d Ab. (**D1**, **D2**) Ly49A epitope interaction-specificity was tested by staining NK cells with anti-Ly49A1 after trogocytosis.

Figure 1

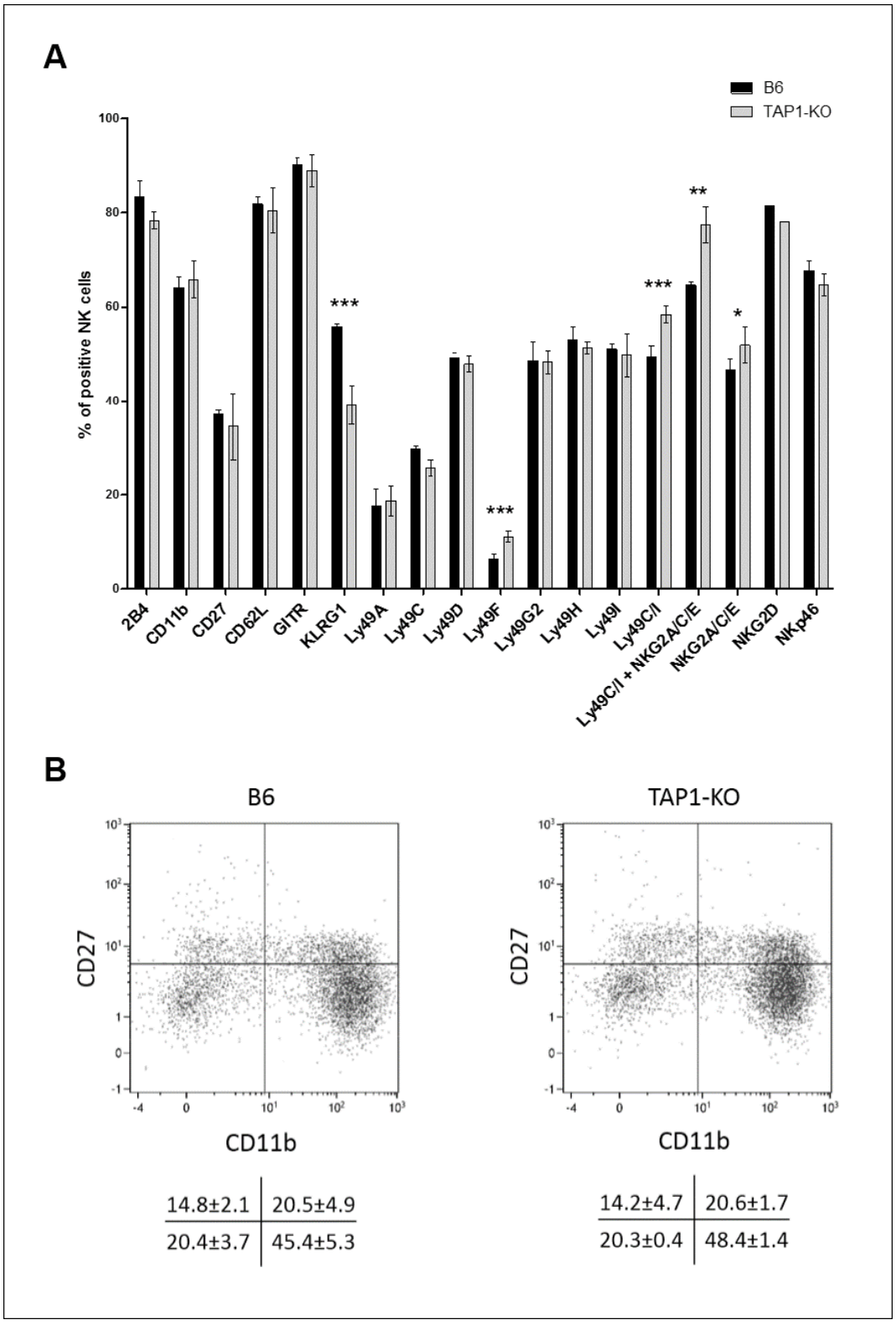

A
B6
TAP1-KO
% of positive NK cells
100
80
60
40
20
0
***
***
**
***
*
2B4
CD11b
CD27
CD62L
GITR
KLRG1
Ly49A
Ly49C
Ly49D
Ly49F
Ly49G2
Ly49H
Ly49I
Ly49C/I
Ly49C/I + NKG2A/C/E
NKG2A/C/E
NKG2D
NKp46
B
B6
TAP1-KO
CD27
CD11b
14.8±2.1
20.5±4.9
20.4±3.7
45.4±5.3
14.2±4.7
20.6±1.7
20.3±0.4
48.4±1.4

Figure 2

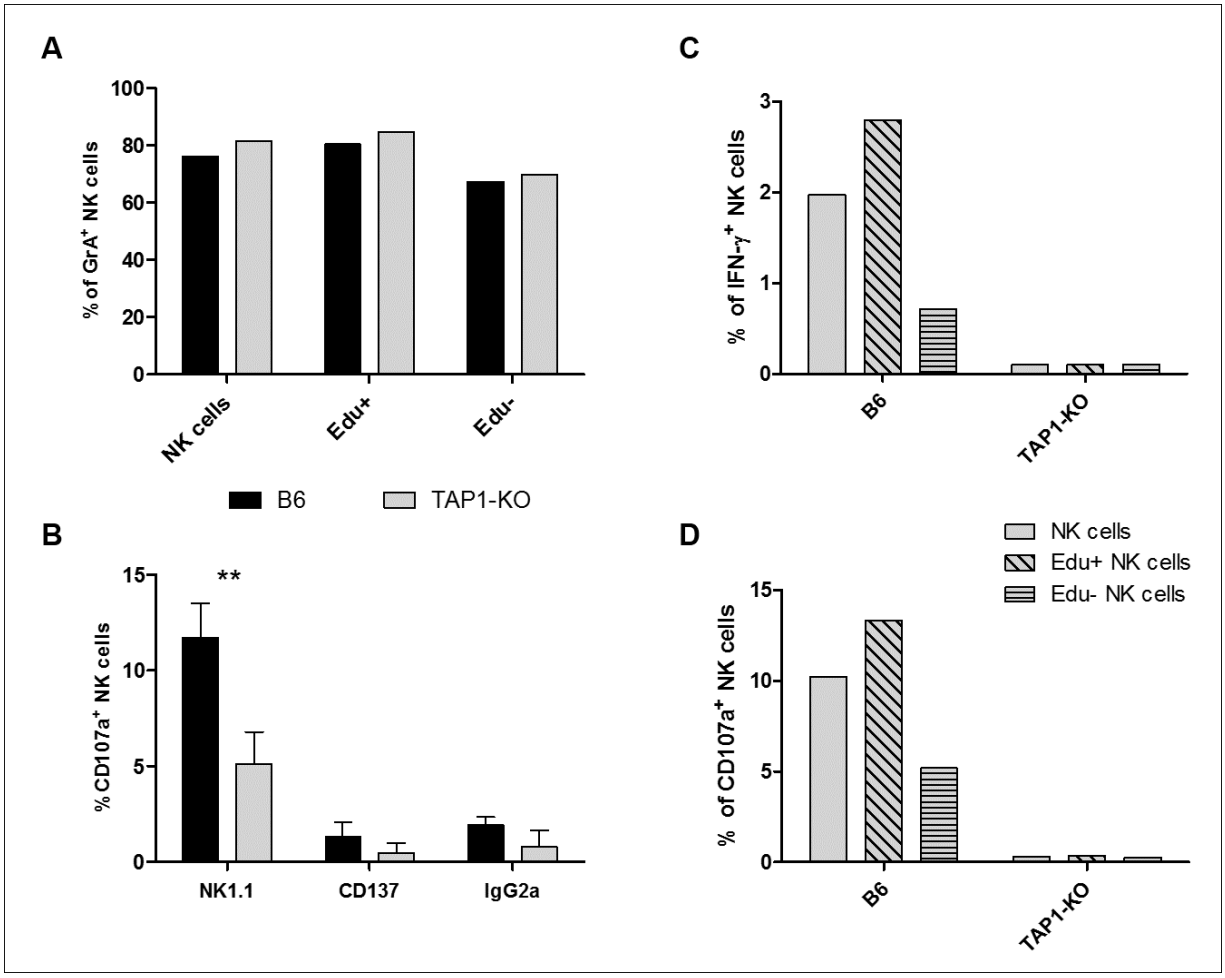
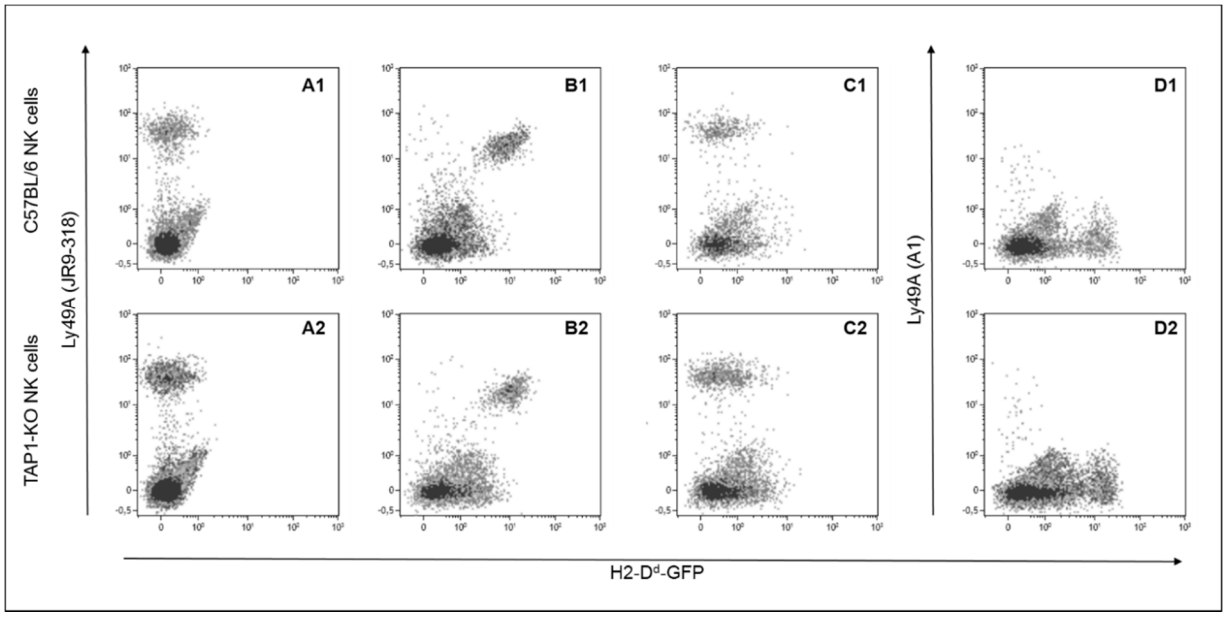


Figure 3



CONCLUSION

Since their discovery in 1974-1975, NK cells became a subject of intensive study. Their capacity to spontaneously kill tumour and infected target cells, secrete a wide range of cytokines and regulate other immune cell functions, made them of great potential importance in several disease treatments. In this study work, we aimed at giving more insight to the above mentioned mechanisms and pathways. Better understanding NK cell education and receptor repertoire formation and adaptation can lead to a safer and wider clinical application.

Taking advantage of our TAP-deficient biologic material stock, and starting collaborations with local and international scientific teams, we were able to shed more light on HLA class I molecules' effect on NK cell phenotype, maturation and functions.

In a close collaboration with the flow cytometry core facility, managed by Nicolaas Brons, we developed an 18-color flow cytometry panel giving us the ability to simultaneously study NK cell functionality as well as IR and maturity markers' expression in TAP-deficient patients compared to healthy donors. In-depth phenotypic analyses showed that in a low surface HLA class I molecules' expression context, NK cells tend to increase the co-expression of their HLA class I receptors (KIR, NKG2A and CD8) especially on the less mature $CD57^{-}$ sub-population. Functionally, TAP-deficient NK cell hyporesponsiveness was confirmed, except for one patient.

Based on these results, and willing to identify the different NK cell maturation and cytotoxicity pathways affected by the low surface expression of HLA class I molecules, whole genome microarrays were performed. Analysis showed that the surface expression level of HLA class I molecules does not seem to have an impact on NK cell maturation from $CD56^{bright}$ to $CD56^{dim}$. A time-series gene profile comparison following 0, 1 and 5 hours of co-incubation with K562 cells demonstrated a different gene expression evolution for each of the two NK cell statuses, leading to an identical profile at the final time point. These results contradict the significant functionality difference between healthy and TAP-deficient NK cells and raise questions on how an identical gene induction can lead to such a significant phenotypic and functional difference. Are the genes induced at the right time in TAP-deficient NK cells, as the similarity between the two statuses was already significant at the 1 hour time point? What about mRNA translation in TAP-deficient NK cells, how is it regulated? Do microRNAs play a role in this regulation, as IFN-γ mRNA is detected at the same level in both NK cell types, but only expressed as a protein in healthy NK cells? A more detailed time-point analyses (between 0 and 1 hour co-incubation) completed by a protein array may contribute to better understand the results we obtained.

In the past few years, several studies mainly by M. Lopez-Botet and K. Malmberg

teams underlined the effect of CMV infections on NK cell repertoire adaptation. A time-stable expansion of $NKG2C^+$ self-specific KIR^+ NK cell population was observed. Among our TAP-deficient patients' cohort, only three presented an NK cell expression of NKG2C (15%-25%). In the light of these results, a collaboration was started with Prof K. Malmberg to whom PBMC derived from two patients were sent (presenting the highest percentage of $NKG2C^+$ NK cells). Experiments performed by Dr V. Béziat demonstrated that contrary to CMV^+ individuals, $NKG2C^+$ NK cells in TAP-deficient patients did not present any skewing toward self-specific KIR expression. Unfortunately as patients' serology is not available, no connection could be done with CMV. Functional tests (rADCC and response towards 721.221-AEH cells) showed that NKG2C does not act as an AR in TAP-deficient individuals, but as an activating co-receptor. Only its combination with a 2B4 or DNAM-1 signal was efficient in activating one of the two studied patients' NK cells.

Our thesis work, combined with our collaborations' results, lead to new fundamental findings in HLA class I molecules' effect on NK cell functional properties and repertoire formation. These findings should allow us to better understand NK cells mechanisms and maybe reconsider some of their features while designing new immunotherapies. The low surface expression of HLA class I molecules seems to have a strong impact on NK cell repertoire and AR functions' pathways. The department of stem cell transplantation (Texas University) in collaboration with Massachusetts Institute of Technology (MIT) and SangamoBioscience are investigating the possibility towards eliminating HLA class I expression and generate universal cells from allogeneic donors. Why not imagining using TAP-deficient NK cells in future immunotherapies? Only the limited amount of TAP-deficient PBMC makes further experiments difficult to perform.

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